BIOCHEMICAL WASTE WATER TREATMENT USING IMMOBILIZED LIPASE ON MULTI-WALL CARBON NANOTUBES

BY

AMMAR JAMIE

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DEANSHIP OF GRADUATE STUDIES

This thesis, written by **AMMAR JAMIE** under the direction his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN CHEMICAL ENGINEERING.**

Dr. Zuhair O. Malaibari
(Advisor)

Dr. Mohammed Ba-Shammakh
Department Chairman

Dr. Muataz A. Ateih
(Member)

Dr. Salam A. Zummo
Dean of Graduate Studies

Dr. Othman C. Al-Hamouz
(Member)

4/3/15
Date

To my mother Raisa, for the early alphabet that she taught me

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LIST OF ABBREVIATIONS

α	Constant defined by Eq 3.12
ω	Mixing speed (rpm)
ϕ	Oil volume fraction in the emulsion
a	Lipase amount (mg)
a_t	Specific free interfacial area (m^{-1})
b	Lipase volume (ml)
E	Free enzyme (mole/total reactor volume) ($mol\ m^{-3}$)
$E * S$	Enzyme/substrate complex ($mol\ m^{-2}$)
E^*	Penetrated enzyme (mole/total interfacial area) ($mol\ m^{-2}$)
E_t	Total active enzyme ($mol\ m^{-3}$)
k	Constant defined by Eq 3.12
k_1	Reaction rate constant (min^{-1})
k_d	Desorption rate constant (min^{-1})
K_e	Equilibrium constant of $E * S$ ($mol\ m^{-3}$)
k_p	Adsorption rate constant ($m^2\ min^{-1}$)
k_{-1}	Reaction rate constant (min^{-1})
k_{cat}	Catalytic rate constant (min^{-1})
m	Constant defined by Eq 3.12
n	Constant defined by Eq 3.12

P	Bulk product concentration (mole/total reactor volume) ($mol\ m^{-3}$)
P^*	Interface product concentration (mole/total interfacial area) ($mol\ m^{-2}$)
S	Bulk substrate concentration ($mol\ m^{-3}$)
Sp_{act}	Specific activity ($\mu mol\ mg^{-1}\ min^{-1}$)
V	Reaction volume (ml)
v_0	Initial velocity ($\mu mol\ ml^{-1}\ min^{-1}$)
CNF	Carbon nano-fibers
CNT	Carbon nano-tubes
FOG	Fats, Oils, and Greases
LU	Lipase Units ($\mu mol\ min^{-1}$)
Mw-CNT	Multi-wall carbon nano-tube
MwCNT-Lipase	Immobilized lipase on multi-wall carbon nano-tubes
v	Rate of reaction ($mol\ m^{-3}\ min^{-1}$)

THESIS ABSTRACT

NAME: Ammar Jamie

TITLE OF STUDY: Biochemical Waste Water Treatment Using Immobilized Lipase on Multi-wall Carbon Nanotubes

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This study investigates the biochemical treatment of oily contaminated waste water using immobilized lipase on the surface of multi-wall carbon nanotubes (Mw-CNT). Multi-wall carbon nanotubes were synthesized locally using chemical vapor deposition (CVD) technique and 0.1 *g CNT/g p-xylene was produced using a floating head catalyst reactor. Produced carbon nano-tubes (CNTs) were treated and functionalized using nitric acid and prepared for lipase immobilization. Results from thermal gravimetric analysis (TGA) showed that about 10% of the CNT was functionalized and oxidized into carboxyl groups. Olive oil/gum Arabic emulsion was prepared and used as a synthetic emulsion to examine the lipase activity and oil hydrolysis reaction and kinetics. By studying different reaction parameters, activation energy for the olive oil hydrolysis by lipase was found to be* 17.26 *KJ/mol and the Michaelis apparent constant* 0.0975 *mol/m*3*. Covalent bonds between the enzyme and Mw-CNTs formed using or-*

ganic cross-linkers and the immobilization process was successfully obtained using an aqueous media solution. Fourier transform infrared (FT-IR) and energy dispersive x-ray spectroscopy (EDX) results showed clear covalent binding between the enzyme and the oxidized Mw-CNT. Thermal gravimetric analysis (TGA) showed that the enzyme loading reached up to 19.5% *wt with very high thermal stability under high temperatures.*

Immobilization of lipase on Mw-CNTs were enhanced the catalytic activity of the enzyme when tested in oil/water emulsions. Lipase activity and enzyme loading had depended on the oxidized Mw-CNT surfaces, cross-linkers type and concentrations, and enzyme amount. The titrimetric analysis of hydrolyzed samples using MwCNT-Lipase (after 1 hr reaction time at $37^{o}C$*) showed an increase in the enzyme activity up to five times compared to the free lipase. The prepared synthetic emulsions were pre-treated using microwave irradiation and the effect of emulsion breaking on the enzymatic treatment was investigated. The exposure periods to the microwave varied from* 60 *to* 180 *Seconds and results showed clear separation of oil layers from water. However, immobilization of lipase into Mw-CNT surface enhanced the hydrophobic properties of the enzyme, thus results had shown that the microwave pretreatment and emulsion separation did not affect the immobilized enzyme activity unlike the free lipase. Oil degradation and hydrolysis using the immobilized enzyme reached up to* 98% *wt. The biomaterial shows high thermal and operational stability and activity when tested in an oil/water emulsions prepared in the lab, and can resist the severe conditions in industrial applications.*

Master of Science

King Fahd University of Petroleum and Minerals

Dhahran, Saudi Arabia

مستخلص الرسالة

الاسم	عمار أحمد جامع أحمد
عنوان الرسالة	المعالجة البيوكيميائية لمياه الصرف الصحي بإستخدام إنزيم اللاييبيز المثبت على أنابيب الكربون النانوية
التخصص	الهندسة الكيميائية
تاريخ التخرج	فبراير، 2015

تهدف هذة الدراسة لمعالجة مياه الصرف الصحي الملوثة بالزيوت بإستخدام إنزيم اللاييبيز (Lipase) المثبت على سطح أنابيب الكربون النانوية متعددة الجدران (Mw-CNTs). تم تصنيع وتحضير انابيب الكربون النانوية متعددة الجدران بإستخدام تقنية الترسيب الكيميائي للأبخرة (CVD) حيث أن الإنتاجية كانت حوالي 0.1 gCNT/g p-Xylene عند إستخدام المفاعل ذو المحفز العائم. الجدير بالذكر أن أنابيب الكربون النانويه المنتجة قد تم معالجتها بإستخدام حمض النيتريك وتحضيرها جيداً لإستخدامها لعملية تثبيت الإنزيم. النتائج التي تحصلنا عليها من جهاز التحليل الحراري الوزني (TGA) تشير إلى أن 10% من وزن أنابيب الكربون النانوية قد تم معالجتها وأكسدتها إلى مجموعات كاربوكسيلية. تم تحضير مستحلب الصمغ العربي وزيت الزيتون وتجهيزه لإختبار نشاط الإنزيم (Lipase) ودراسة خواصة وآلية تفاعله مع الزيوت. وبعد دراسة عدة عوامل للتفاعل، لقد توصلنا إلى أن طاقة التنشيط

للتفاعل المذكور أعلاه وصلت إلى 17.26 KJ/mol وثابت ميكائيلي 0.0975 mol/m3. لقد تم إستخدام أنابيب الكربون النانوية لإنشاء روابط تساهمية مع الإنزيم حيث تم تثبيته على سطحها. النتائج التي تحصلنا عليها من جهاز الأشعة تحت الحمراء (FT-IR) وجهازتشتت الأشعة السينية (EDX) تشير إلى نشوء رابطة تساهمية قوية بين الإنزيم وأنابيب الكربون النانوية. بعد تحليل العينات بإستخدام جهاز التحليل الحراري الوزني، تم إكتشاف أن 19.5% من وزن العينة بعد عملية التثبيت يرجع إلى وجود الإنزيم على سطح أنابيب الكربون النانوية. عملية تثبيت الإنزيم على سطح الأنابيب النانوية زاد من نشاط الإنزيم عندما تم إختباره في مستحلب زيت الزيتون. ولقد تم التوصل إلى أن كمية الإنزيم المثبت تعتمد على كفاءة تحضير الأنابيب النانوية، ومساحة سطحها، ونوع المركبات الرابطة المستخدمة (Cross-Linkers) وتركيزها، وكمية الإنزيم المضافة. عند تحليل الإنزيم المثبت بإستخدام المعايرة بعد تفاعله مع المستحلب (زمن التفاعل ساعة واحدة، و 37 درجة مئوية)، تُوُصِّل إلى أن نشاط الإنزيم المثبت يزيد خمسة أضعاف الإنزيم قبل التثبيت على الأنابيب النانوية. الجدير بالذكر أنه قد تم أيضاً دراسة تأثير معالجة المستحلب بموجات المايكرويف قبل تعريضة للمعالجة الإنزيمية. تعريض المستحلب لموجات فرن المايكرويف لفترات زمنية تتراوح بين دقيقة وثلاث دقائق أدى إلى فصل طبقات الزيت من الماء داخل المستحلب. لكن وبعد الدراسة المستفيضة، أظهرت النتائج أن الإنزيم المثبت على أنابيب الكربون النانوية لا يحتاج ولا يتأثر بالمعالجة المبدئية بإستخدام المايكرويف، حيث أن عملية التثبيت على سطح الكربون النانوي قد أضافت خصائص وميزات إضافية للإنزيم. نسبة معالجة المياة الملوثة بالزيوت بإستخدام الإنزيم المثبت وصلت لحوالي 98%. هذة الدراسة أثبتت أن إستخدام طريقة تثبيت الإنزيم تضيف إستقرار وثبات للإنزيم وتزيد من نشاطه عند إستخدامه لمعالجاة مستحلبات الزيوت التي تم تحضيرها في المعمل، ومن

المتوقع أن يكون له نفس الأثر على المياة الملوثة الحقيقة في مخلفات المصانع والبترول.

ماجستير العلوم

جامعة الملك فهد للبترول والمعادن

الظهران، المملكة العربية السعودية

CHAPTER 1

BACKGROUND AND RATIONALE OF THE STUDY

1.1 Introduction

Currently, as the world witnesses vast expands in all major industrial fields to satisfy the famine of human needs, issues related to the huge mass production such as waste handling and pollution management has become a significant problem. Food processing plants, slaughterhouses, restaurants, oil mills factories, and even daily domestic waste water are all considered major sources of oily and fatty polluted waste water. With daily increasing demand and consumption, treating all amounts of polluted waste water has became a real issue to the waste water treatment plants. However, during the recent years there are many methods and processes that have been developed to deal with industrial and sewage waste water. Nevertheless, looking for coast-efficient, and environmentally friendly techniques to tackle the problem is not an easy task. Hence, significant amounts of money and research has been allocated to

find alternative precursors that can give desirable properties of materials used in such special applications.

1.2 Rationale of the Study

Biotechnology is playing a major role in medical, pharmaceutical, genetic, food industry, and other applications nowadays. Not only because it is a new nature-friendly technology, but also because it has the ability to treat problems caused by traditional technologies in a safe efficient manner. Sub-fields such as environmental biotechnology has started to have a great interest because of the green nature associated. On the other hand, nano-structured carbon materials like carbon nano-tubes (CNT), carbon nano-fibers (CNF), graphene, and graphite have also witnessed, in the last decade, great implications as: (1) a nano-layers coats; (2) electrical, optical and mechanical nano-devices applications; (3) and nano-structured biological materials. Because of its unique physical, electrical, and thermal properties, carbon nanotubes (CNT) has become one of the most interesting materials as discussed. Researchers and scientists are allocating considerable effort and resources to investigate and develop new technologies for the synthesis and enhancement of this material. Combining these recent booming technologies (Nano and Bio)-technology in an environmental application will be even more interesting, challenging and promising. In this study, the main idea is to use an enzyme (lipase) immobilized on carbon nanotubes (CNT) to treat waste water contaminated with fats, oils, and grease (FOG). Which will be a preliminary atep to use this combination for further industrial applications.

Lipases is a well-known hydrolysis enzyme for its ability to degrade complex oils and fats into free fatty acids that are easier to be handled. It has been used for many decades in most biochemical applications including medical, pharmaceutical, detergent, and environmental. However, its applications is restricted by its week physical and thermal properties. Thus, CNTs will be used to enhance the physical and operational properties of the lipase for water treatment. Hence, the immobilization process is expected to increase the stability and the thermal operability of the enzyme. Additionally, pretreatment methods such as microwave treatment which is known as an efficient method for the waste pretreatment and handling, will be investigated. Of primary interest is, its effect on waste water and emulsions breaking and degradation of oily pollutants.

1.3 Source of the Problem

Fats, oils, and grease, referred as 'FOG', are one of the main water pollutants coming from food industries, restaurants, slaughterhouses, lather processing units, and dairy waste water [1–4]. Many problems face waste water plants when handling the oily or fatty polluted water. Mainly, waste water contains other pollutants including salts and minerals, not only being considered as hazardous materials, but also because they work as emulsifiers that help in forming different complex water/oil emulsions [5]. Moreover, because of the heavy viscous property of the FOG, it clogs the networks in waste water treatment plants causing additional maintenance concerns for the industry. Furthermore, the floatation of oils on the water/air interface causes real issues

in activated sludge processes and biological treatment, since it limits oxygen transfer into the water to activate the biomass [6]. Concurrently, microorganisms and bacteria used in these processes adsorbs the oils and grease and it cause it to float to the surface reducing the efficiency of treatment. Additionally, fatty acids resulted from FOG esterification and hydrolysis inhabits the bacteria and microorganisms activity [7]. Beside these severe treatment technology concerns, FOG causes serious health and environmental problems by blocking sewer pipes and reducing its diameter, causing consequently flooding and attract pathogens and vermin [8].

1.4 Objectives of the Study

The main goal of this study is to synthesize an immobilized lipase on multi-wall carbon nano-tubes and use it in-line with the microwave pretreatment for the treatment of oily waste water. Specifically, the main objectives of the study are:

1. To produce high quality multi-wall carbon nanotubes (MW-CNT) with high aspect ratio using chemical vapor deposition reactor.

2. To treat the produced CNT and functionalize its surface with carboxyl functional groups using acid oxidation.

3. To study the kinetics of olive oil emulsions hydrolysis using free lipase and investigate the effect of different reaction conditions.

4. To enhance the thermal, physical, and chemical properties of Candida Rugosa lipase by immobilization on the surface of multi-wall carbon nanotubes.

5. To use microwaves as an effective pretreatment method for the oily waste water prior the enzymatic treatment.

Research Question

Does Immobilized lipase on MW-CNT assisted by microwave pretreatment have significant effect on oil degradation in waste water?

1.5 Enzyme Immobilization: Properties and Benefits

1.5.1 Immobilization Process

The term "enzyme immobilization" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" [9]. Mainly three components defines a system of immobilized enzyme: the enzyme to be attached or confined, the support (matrix) that would be used for the immobilization, and the immobilization procedure. It is very clear that the immobilization of a biocatalyst had been developed through the years in three main steps: Firstly, the empirical stage where the immobilization of microorganisms at the early nineteenth century years was applied to small scale industrial experiments. At that time, the microbial production of vinegar started by dropping alcohol solutions over wood shavings grown with bacteria and some developments was made on waste water clarification using trickling filter. The

second step, which considered as the modern enzyme immobilization start, begun at the 1940s but allot of that early work was not considered and gave an attention by scientists. That was because it was recent area at that time and most of the work was published in other disciplines journals. But, the base of the recent industrial technologies was developed during the 1960s. At that time there was a booming increase in research and publications related to this field. During the second stage, immobilization methods was focused on single enzymes attachment for L-amino-acids production and glucose isomerization. By the 1970s, a complex immobilization methods and enzyme attachment procedures started and the third step appeared. Living cells were developed during this step and it witnessed involving more than one enzyme in reactions with cofactor regeneration. During this period many enzymes have been applied in organic synthesis as a bio-catalysts. Including most of the recent enzymes, hydrolytic esterification enzymes like esterases and lipases have been regularly involved in many research work and bio-applications. This is due to the wide-ranging substrate specificity and the fact that they have excellent enantioselectivity. These enzymes was used specifically in some applications and studies related to esters synthesis, fatty acids production, and making an optically active alcohols. However, they still have some limitation, importantly, including its low stability and activity in most organic media. Recent research and studies interests are to develop an immobilized enzymes that have better stability and activity in organic solvent in order to use it for severe applications such as oily waste water treatment [9].

1.5.2 Benefits of Enzyme Immobilization

Attaching enzymes to a certain support has many advantages as mentioned by previous studies [9–13]. Figure 1.1 illustrates two major merits for enzyme immobilization: (1) Support matrix has chemical characteristics and mechanical properties such as strength, temperature handling, surface area, thermal and electrical conductivity and the most important is the ability to be fabricated in different reactor processes including packing, fluidization, immersing and recycling; (2) Enzymes have another reactive and biochemical properties which control the catalytic process and the type of kinetics and it determines the final products and outcomes. Combining both properties provide many merits to the whole process. Hence, the operational stability is the most important key factor for any catalyst used in the industry, the support increases the durability and the mass transfer effect maintain the high efficiency for the immobilized enzyme. Furthermore, the immobilization method controls the yield and the reaction performance since it affects the chemical characteristics of the enzyme [9].

1.5.3 Enzyme Immobilization Methods

Many immobilization processes and methods was developed in recent years to enhance reliability, durability, and stability of the enzymes. The preference of one method to the others depends on the application in which the immobilized enzyme would be used for. Mainly, these methods could be classified as reversible and irreversible. In irreversible methods shown in figure 1.2, the enzyme is attached to the support and there is no way to reverse the process again to get the free enzyme. These

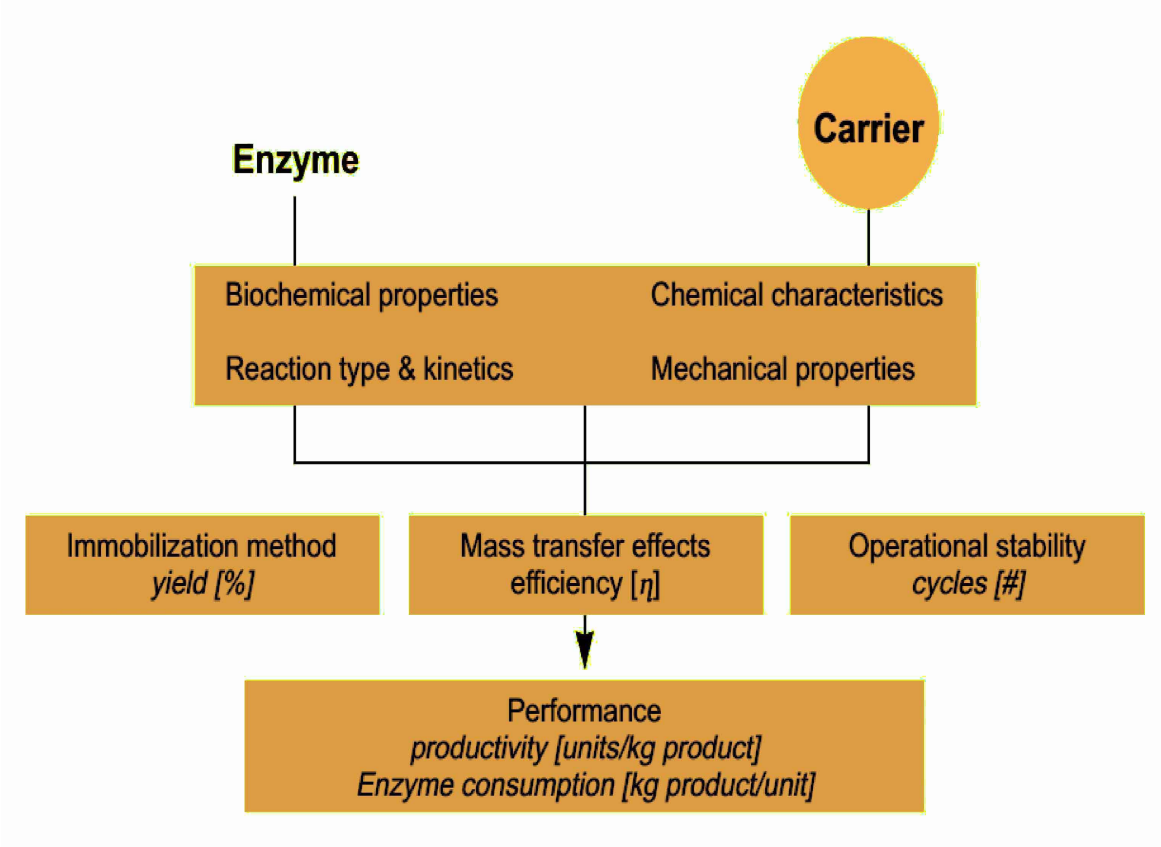

Figure 1.1: Enzyme Immobilization Properties and Benefits [9]

methods include: entrapment, cross-linking, encapsulation, and covalent binding. On the other hand, the reversible binding shown by figure 1.3, refers to the process in which the enzyme could be detached after immobilizing on to the support and it includes: adsorption, ionic binding, affinity binding, chelation or metal binding and disulphide bonds [9].

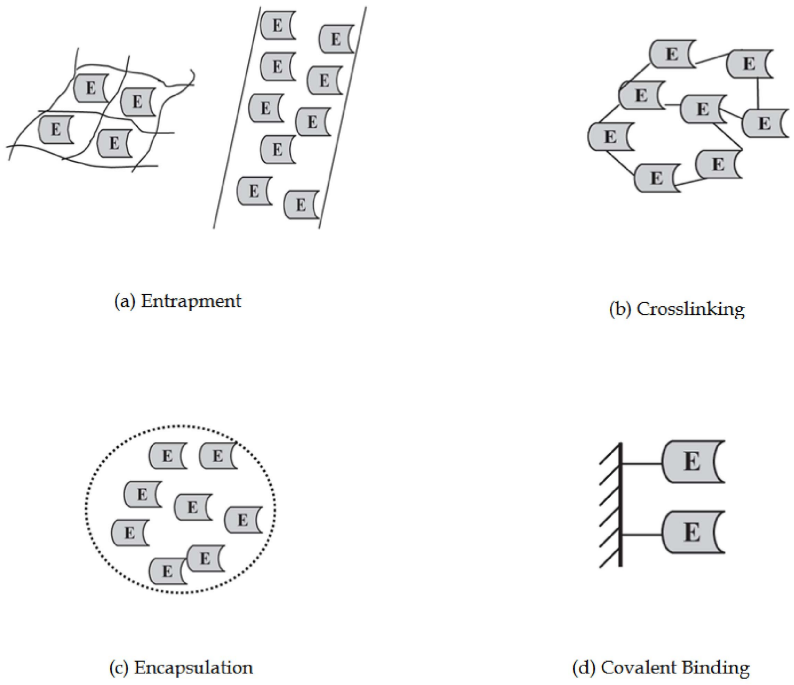

Figure 1.2: Irreversible Immobilization Methods, (a) Entrapment, (b) Crosslinking, (c) Encapsulation, and (d) Covalent Binding [9].

1.5.4 Supports and Matrices Used for Immobilization Process

The properties of the support matrix is very important in determining the properties of the immobilized enzyme. The Ideal matrix would have physical properties, bio-

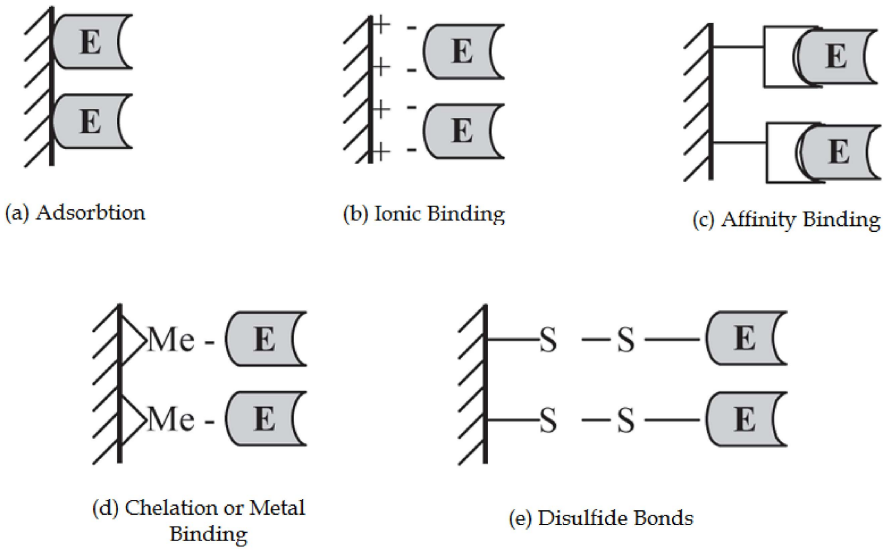

Figure 1.3: Reversible Immobilization Methods, (a) Adsorption, (b) Ionic Binding, (c) Affinity Binding, (d) Chelation or Metal Binding, and (e) Disulfide Binding [9].

compatibility, inert, resistance to microbial attack and of course low cost. Different materials had been used as supports for lipases including organics and inorganics according to its chemical composition. Organic supports include Polysaccharides (such as cellulose, agar, and chitin), proteins, and carbon. All of these materials considered natural polymers and supports for lipases. There are many synthetic polymers used as matrices such as polystyrene, polyacrylate, and polyacrylamides. Inorganic supports were also used to immobilize enzymes and there are many interesting materials which has good properties make it compatible with the immobilization applications such as bentonite, silica and glasses (controlled and non-porous) and metals [9]. Recently, use of nanomaterials as a supports for the enzyme immobilization has received significant attention. It is well known that many nanomaterials have very large surface area which can provide large enzymes loading and also it has many physical, mechanical

and electrical properties. All this properties puts the nanomaterials it in the forefront of the global research interest for such interesting applications [14, 15]. Carbon nanotubes, as a nano-scale one dimensional tubular material has a graphitic structure, considered one of the most famous nanomaterials that could be used as a support for enzymes immobilization and its applications. Considering its high aspect ratio and significant mechanical properties it is recommended as a very good support for enzyme loading [10].

1.6 Microwave Pretreatment and Emulsion Breaking Effect

The development of microwaves for heating applications had increased since the Second World War. This is due to the nature of microwaves in providing rapid, efficient heating. Wood drying, food processing, rubber and plastic treatment, and drying and ceramics preheating. Mainly, we mean by microwave irradiation the electromagnetic radiation with a frequency range of 300 MHz to 300 GHz. Generally, 2.45 GHz is the frequency range for both industrial and domestic microwave ovens equivalent to a wavelength of 12.2 cm. Although microwaves raise as an efficient heating process, however some materials cannot be heated by microwaves. All materials are classified into one of the following three groups: absorbers, insulators, conductors [16]. As shown in figure 1.4, absorber materials named as dielectrics, hence, microwave heating is known as dielectric heating. This kind of materials have two major properties [17]:

(1) they have a small number of free charge carriers. Applying an external electric field makes a small amount of charges passes over the material; (2) the atoms or molecules consisting of a dielectric shows a dipole agitation. The dipole is where two equivalent and opposite charges alienated by a finite space. An example of this is water, the stereochemistry of covalent bonds in its molecules gives water molecule a dipole movement. Water is a classic example of the non-symmetric molecule [18]. The application of an external electric field can alter the electrons and cause rounding of the non-polar molecules which can induce a transitory dipole agitation. This agitation dissipate friction inside the dielectric and subsequent heat generation occur [16].

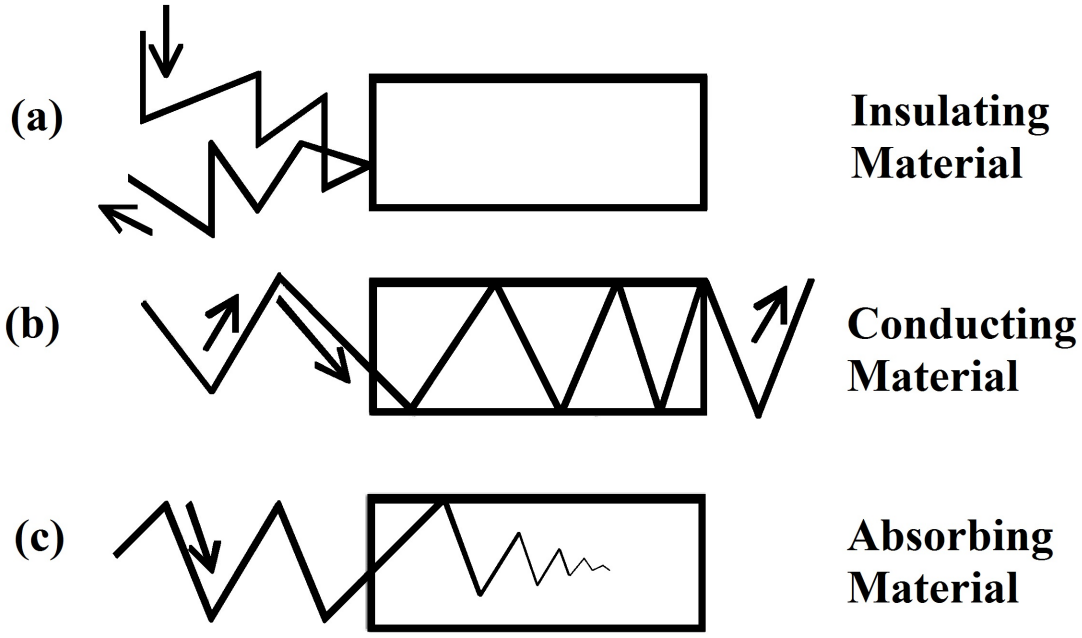

Figure 1.4: Materials classification according to their interactions with microwaves: (a) Insulator, (b) Conductor, and (c) Absorber

Dielectric materials interaction with the electromagnetic radiation causes energy absorbance when we use microwaves. Lossy dielectrics is the only materials known

with absorbing microwave energy and sink it as heat. The electric field generated by microwaves results in a rapid alignment and realignment of dipoles in a polar solvent like water. Repeating this process continuously makes the friction that results the heat. Microwave heating has the hotspot phenomenon because it initiates a of very high temperature regions due to the non-uniform heating [19]. The nonlinear dependence of the thermal properties on temperature causes this kind of thermal instability [20]. Because of the vertical waves within microwave ovens would be formed during heating, some spots got exposed to higher energy than others. This one with the non-linear dependence results in an increasing heating rate in these particular points. To insure an efficient and uniform heating, oven design should be considered as controlling factor to utilize this hotspot phenomenon. A big advantage for the microwave heating over other traditional methods (bulk heating in furnaces) is the capability of selective and efficient heating, hence, no energy is lost to the sample "bulk heating". Currently, investigation and research is going to find applications in many fields where the microwave energy advantages has a significant saving in energy costs, environmental treatments methods, and process times. In comparison with conventional heating methods, microwave irradiation has additional advantages as follows:

1. Has a higher heating rates.

2. Practical equipment size.

3. Selective heating property.

4. Efficient heating process with great control.

5. No direct interaction between the source of heating and the material.

CHAPTER 2

LITERATURE REVIEW

2.1 Waste water Treatment Technologies

There are many methods and techniques that are used in waste water treatment. The preference of one to the others depends on many factors including the source of the water, the contents of the pollutants, and the usage of water after treatment. The removal of oil and the treatment efficiency is determined by the concentration and physical properties of the oils existing and its drop size [5]. Main methods for the treatment of such polluted waste water includes physical, chemical, biological and other combined process.

2.1.1 Physical Treatment Methods

Many physical treatment processes such as flotation, sedimentation, evaporation, filtration, centrifugation, and membrane technology, are used in oily waste water processing.

Floatation is one of the famous techniques when come to organic pollutants treat-

ment. Methods like dissolved air floatation (DAF) and induced air floatation (IAF) considered as an important methods in waste water handling and oily waste water treatment specifically.However, due to the nature of the waste water that contains other salts, metals, organic polymers, and inorganic solids, different complex types of emulsions are formed. These stable oil/water emulsions makes allot of issue and limitations to the use such techniques in oily waste water handling. Mostly,flotation is used as a pretreatment stage before the biochemical treatment of the oily waste water [21–23].

Sedimentation using big tanks or evaporation of water in open large ponds considered as a well-known methods for the concentration of oils and organic matters for further treatment. But none pleasant odor coming from these widely open systems and pathogens and vermin that attracted by evaporation ponds is considered real concern for the disposal in such a way. In addition to, other environmental aspects related to the later disposal and landfill to outdoor environment puts real concerns and limitations about these traditional methods.

Filtration and centrifugation are important methods for FOG removal through exclusion and phase separation, respectively. However, the viscous and sticky nature of these types of pollutants make a real difficulties with the repetitive use of devices and equipmentin such methods of treatment. FOG clogs and block the filtration media and sticks to the centrifuges walls during the separation process adding more complications to the process [24]. Methods as sedimentation, evaporation, filtration, and centrifugation are traditional and also limited by the formation of oil/water emulsions

in most cases.

Membranes are one of the famous separation techniques that have been used recently for pollutants separation from the waste water. There are many types of membrane separation technologies such as reverse osmoses, ultra-filtration, micro-filtration, and nano-filtration used in the separation of oils form water. Although, membranes are relatively expensive and needs adjusted conditions, but it is very efficient as separation processes and could be used for the hard stable emulsions treatment [4].

As discussed, all these techniques mentioned above, are unable to efficiently remove and treat all the organics contained in the oily polluted water. Often, combined physical processes joined with the coagulation, flocculation or adsorption technologies results in high efficiency removal of oils, fats, grease, and all other organic and inorganic pollutants.

Tarek Aziz et al.(2011), performed experiment using grease abatement device with two compartments and computational fluid dynamic model (CFD) to study the effect of internal geometry modification and the hydraulic retention time (HRT) on the FOG removal [25]. They provided a complete simulation model for their results and the optimum HRT was found to be 1 h with removal efficiency about 97% [25].

2.1.2 Chemical Treatment Methods

There are many chemical treatments that are applied to waste water processing. The preference of one to the others depends on many factors including the source of the waste water, pollutants and chemical composition, and the target of the treatment and

use of treated water. Some of the most famous chemical treatment methods includes chemical precipitation, chemical oxidation, electrochemical process, photo-catalytic treatment, fenton process, treatment with ozone at room temperature ionic liquids, and demulsification [26].

2.1.3 Biological Treatment Methods

After appropriate pretreatment of the oily waste water, it is introduced to a biological treatment using micro-organisms to degrade the organic content. Because biological treatments have the advantages of being environmentally friendly, having low cost, and mostly reliable, it has been used widely in the treatment of the waste water. Mainly, two types of biological systems are used, aerobic and anaerobic [24, 27].

Anaerobic Processes

Organic pollutants, represented by chemical oxygen demand (COD) and biological oxygen demand (BOD_5), could be degraded into small amount of sludge and biogas by applying anaerobic process. As reported by Chowdhury et al.(2010), with no need for aeration (oxygen), it is costly effective, low space required, produce valuable methane and a little amount of biomass and sludge [27]. Demirel et al.(2005), recommended the anaerobic treatment as an efficient diary waste water treatment process.It is preferred because of its advantages over the aerobic treatment methods in handling industrial waste water, particularly, from agricultural processes which contains considerable organic content. COD removal efficiency as reported at hydraulic retention time was between 78 and 92% in a laboratory scale filter reactor and the temperature

effect was negligible in the range of 21 to 30^oC [28].

Chowdhury et al.(2010), studied and reviewed many methods including anaerobic filters. He mentioned that many studies recommended fluidized bed and filter reactors considering th effect of temperature in the COD reduction and the gas production [27]. Other studies done by Prasertsan et al.(1994), reported that the organic loading rate (OLR) had significant effect on the degradation of the organics which affects the production of the biogas. They reported 84% COD removal when they conducted the experiment with OLR of 0.3 $kg\ COD/(m^3\ day)$ with maximum hydraulic retention time (HRT) of 36 days [27].

Up flow anaerobic sludge bed (UASB) is an aerobic treatment method where a blanket of granular sludge suspends in the tank. Flowing upward through the blanket, the waste water degraded by the anaerobic microorganisms. UASB considered as one of the most efficient and famous biological methods which had been recently applied. Paraskeva and Diamadopoulos (2006) reported that, in recent laboratory –scale up-studies using UASB reactors and starting by 22.6 *to* 97 g/L COD,the treatment removed 70 *to* 80 % of the organics. They changed the organic loading rate (OLR) between 0.83 *to* 21.9 kg COD $/(m^3day)$, keeping an average of 5 kg COD $/(m^3day)$. Hydraulic retention time was changed between 2 to 5 days. The initial effluent was diluted before adding nutrients to adjust the alkalinity. As HRT is increased to 25 days, COD was reduced by 87.9% and the produced Methane was proportionally increasing with the COD reduction reaching a rate of 0.30 *to* 0.35 $m^3\ CH_4/kg$ COD removed. The use of anaerobic sequencing batch reactor for treatment reduces COD contents

up to 80% with 3 days HRT and an average of 5.3 kg COD m^{-3} day^{1} OLR [24].

Rajesh et al.(2008), they used solar anaerobic integrated process to treat the oily waste water. They concluded that the anaerobic treatment is more efficient compared to aerobic methods, hence, the HRT was 14 to 20 h for the anaerobic compared to only 5.9 h for the anaerobic. And the integration of the solar photo-catalytic method with biological method resulted in 96% and 95% removal of BOD_5 and COD removal, respectively [29]. Although of the great advantages mentioned above, yet some issues related to the use of the anaerobic treatment of industrial waste waters appears. The high content of fats in the waste water arises as a great challenge for such processes.When we use processes like UASB, flotation of the sludge is considered one of the problems that we face [29, 30].

Another issue is that, at low temperatures and with the high fat content an agglomeration of solid bulks starts to be formed. This one causes operational issues and it clogs the waste water networks and equipment and forms unpleasant odors to the environment. Moreover, for its floating nature, grease and oils limits the transfer of substrates to the biomass on the surface of the sludge reducing the conversion rate [30].

Aerobic Processes

There are many types of aerobic processes in which aeration is an important part of the process such as activated sludge, trickling filter and lagoons, and rotating biological contactor. Activated sludge is the conventional method for water treatment in which oil skimmers are used to skim the separated oil before the later biological

treatment [27]. Fakhru'l-Razi et al.(2009), reported that the removal efficiency for the activated sludge reached about 99% when treated for 20 days solid retention time in sequencing batch reactor (SBR). They reported an efficiency of COD removal from 30% up to 50% in acclimated sewage sludge using sequencing batch reactor with different sewage an produced waste water amounts [26].

Kalyanaraman et al.(2012), studied the effect of the food to the micro-organism ratio (f/m) on the COD and BOD_5 removal efficiency in activated sludge experiment. They used a vegetable based fats in their study and the optimum f/m ratio was found to be 0.15 for an efficient degradation. About 89 and 97% of COD and BOD_5 removal efficiencies, respectively, was reported for 1 day reaction time [31]. Alade et al. (2011), reported that oils and greases treatment processes including aerobic, anaerobic, or in combination with physico-chemical treatment alternatives, requires huge capital investment, skilled labor force, and intensive energy requirements [32].

2.1.4 Enzymatic Wastewater Treatment

The use of enzymes and its related processes can be traced back to the earliest beginning of civilizations. Nowadays, about four thousand enzymes are used and well known, and out of these, approximately two hundred are used commercially. Most of commercially used enzymes are from microbial sources [33]. Since there are an increasing improvement and better understanding of biochemical synthesis, fermentation technologies, and biotechnology processes, affordable amounts of enzymes can now be produced. Likewise, many advancements in enzymes applications have sig-

nificantly expanded request. Moreover, because of the many different reactions that enzymes can catalyze, the demand for enzymes used commercially is continuously increasing [34].

2.2 Synthesis of Carbon Nanotubes and CVD Technique

Carbon nanostructured materials have great research interest and developments recently. Considerable research efforts and industrial application was focused for the commercial production and implementation of these type of nano-materials. Through the last two decades, discoveries like carbon nanotubes (CNT), buckminsterfullerene, carbon nanofibers (CNF) enlarged this area rapidly and continuously, opening a total new area of research [35]. Carbon nanotubes (CNTs) and carbon nanofibers (CNFs) arises in the forefront of the revolutionary nano-materials in nanotechnology developments. Because of their electronic, mechanical, optical and chemical characteristics, they open the way to many future and industrial applications. These unique properties could be tested even in a single nanotube and nanofiber [36, 37]. Applications including molecular electronics, nano-mechanical devices and equipment, transistors, capacitors, and nano-composite materials are all possible to have great leaps toward high efficiency using such materials. Iijima (1991), discovered a thin and long carbon rolled sheet which have the nanotubes shape when he was analyzing carbonaceous groups by transmittance electron microscopy (TEM). He used arc discharge method

to, coincidently, synthesize the first nanotubes ever observed. After further analysis using TEM imaging he observed the uniform long tube bundles in his samples [38]. CNTs has a length varies from few nanometers to several micrometers, and its diameter approximately is between 2.5 and 30 nm. Ebbesen and Ajayan (1992) they observed that, the increment of pressure in the chambers of an arc exceptionally improved the carbon nanotube yield at the cathode of the graphite. Following his glorious discovery, Iijima and Bethune (1993) have synthesized CNTs with one nm diameters, using the arc-discharge [39, 40].

Smalley et al. (1996), reported a new preparation method using laser vaporization of graphite to synthesize an unusual homogenous diameter single walled nanotubes which had a tendency to be formed in aligned nanotube bundles ' rope ' [41]. Yacamán et al. (1993), using chemical vapor deposition (CVD), they made a large achievement by synthesizing CNTs in large quantities and with good quality. Since that time, investigations in synthesis and application on the CNTs have been actively started and got great interest in all research area [42].

2.3 Lipase Immobilization

As reported by Brena and Batista (2006); the usage of immobilized enzymes onto other materials goes back to the earliest of 19^{th} century [9], when immobilized enzymes were first prepared intentionally. Enzymes, specially lipase, are considered highly selective and bio-compatible material for the modification of nano-scale materials surfaces. Many studies had been done in immobilization of enzymes to different supports to

enhance their stability, durability and process alternatives [9, 43]. Tischer et al.; had mentioned many methods and techniques to immobilize the enzymes which could be categorized into the following: i) Support attachment which could be physical, ionic, or covalent in its nature; ii) Crosslinking of enzyme, using a bi-functional substance, to prepare carrier less macro particles; iii) Encapsulation in a polymeric network such as hollow fiber membranes, a silica sol-gel, microcapsules or organic polymer [13, 43].

Materials used in the immobilization process is classified into two main categories. Firstly, organic polymers which can be natural as polysaccharides, portions, and carbon or synthetic polymers as polystyrene, polyacrylate, and polyacrylamides. Secondly, there are the inorganic materials such as silica and bentonite or could be handled materials like controlled pore metal oxides, and glasses [9]. Soares and Castro in 1999 used controlled pore silica (CPS) as one of the inorganic types of enzymes supports. It showed that the attachment of lipase to this support increased the enzyme ability to withstand 10^oC higher than free enzyme which is unstable above 40^oC and has low relative activity [44]. But, because of its high surface area, mechanical strength and thermal and electrical conductivity, carbon nanotubes (CNTs) are considered one of the most recent targeted material for enzymes immobilization and biosensors and bio-catalytic applications [45].

Wang et al. in 2003 used CNT's in immobilizing enzymes, followed by Lee et al. and Pavalidis in 2010. They used single and multi-wall CNTs in their research on lipase immobilization [10,45,46]. Lee et al. they discovered that the immobilizations of lipase on SWCNT is more efficient in the existence of ionic liquid than those in buffer

solutions. At the same time, the use of non-covalent bonding via an anchoring 1-pyrenebutyric acid N-hydroxysuccinimide (NHS) reagent is more efficient than simple physical adsorption [45]. Other studies done by Ji and Peijun (2010) on covalent attachment of lipase to multiwall CNTs shows good results in term of thermal stability, durability and product selectivity. Because of the high thermal conductivity, the attachment of lipase covalently to Mw-CNT reserved up to 82% of the free enzyme activity and the conversion of immobilized enzyme was higher and more stable than for free enzyme in the range from 40 to 55^oC for the reaction temperature [47].

2.4 Waste Water Treatment Using Microwaves

Microwave (MW) irradiation is an alternative method to conventional thermal treatment suitable to produce rapidly focused direct heat with low transmission energy losses. The existence of so-called non-thermal microwave effect is due to the change in dipole orientation of polar molecules.

Kliala (1983) and Wolf (1986) believed to be the first scientists that had suggested the idea of microwave emulsions treatment in their patent applications [48, 49]. Following his patent award, Klaila conducted several field tests, with a 50 kW microwave generator, which was equipped with wave guides and a microwave power monitor. Recently, active research and development work on microwave demulsification technology for applications in chemical plants is carried out by many researchers [50–54]. In Kansas, Fang et al. (1995) used a large storage tank (ten ft in diameter and ten ft high) to treat 120 bbls of slop oil. The emulsion was 50% oil, 27.5% water and 22.5%

sediment. They applied 228 kWh of microwave energy continuously, the temperature of the top portion of emulsion reached approximately 100^oC and the emulsion was separated to oil and water layers. Similar results were obtained in field test in Louisiana for a tank contained 188 bbls of crude oil water emulsion, but this time the tank had a height of 15 ft and 10 ft diameter. After exposure for 18.2 hours and using 417.5 kWh of radiation power with the emulsion, 146 barrels of oil was sepa¬rated from 42 barrels of clear water [51].

In 2002 Camila et al; studied the effect of microwaves on different oil water emulsion percentages. All cases showed that separation degree of water had increased with the increase of the exposure time. However, increasing the amount of NaOH in the emulsion lowered the percentages of separation. The recoveries in emulsions have a maximum of 60% when NaOH added, whereas mixtures without NaOH reached up to 80% for the water separated. Furthermore, they observed the initial ratio of the water to oil controls the maximum exposure time, hence, the maximum exposure time for 30 oil to 70 water emulsions is 300 seconds, while it was 240 seconds for 17 to 83 oil/water emulsions [52].

Vladana et al. (2005) studied the effect of microwave in line with freeze/thaw method in emulsion breaking and demulsification. By varying the amount of oil from 0.1 to 30% in the emulsion and applying techniques above using microwave oven (95^oC, 800 W, and 2450 MHZ), they found that applying MW radiation made the molecules excited, this excitement results in super-heating and high rate of reactions. The speed and the efficiency of the demulsification process was increased by the use

of microwave radiation, and it is recommended as an efficient method, regarding the thawing technique used in their experiments. Finally, the efficiency for oil water emulsions up to 30% oil was tested to be above 90% [53].

Coutinho et al. (2010) patented a method of microwave emulsion treatment by optimizing many parameters such as water amount, content of salts, value of pH for the aqueous phase, initial temperature, microwave power, final temperature and drop size distribution to be adjusted in the industrial plant. Each 80 ml of the emulsion was exposed to 1400 W microwave power for 15 min and then followed by 10 min sedimentation time, the amount of water separated by the well-known Karl Fischer titration. After detailed study, the efficiency Coutinho found to be around 25% of separated water and the ideal pH was found to be from 7 to 9.5. The efficiency of the process is increasing proportionally with the increase of temperature. above 90^oC at 130^oC. It should be noticed that high temperature requires intense microwave power [54].

CHAPTER 3

KINETICS STUDY OF OLIVE OIL HYDROLYSIS USING CANDIDA RUGOSA LIPASE

Abstract

In this study, the kinetics of olive oil hydrolysis by lipase enzyme was investigated. Different reaction conditions was varied and tested, since the effect of temperature, mixing speed, pH, enzyme loading, and substrate concentration on the rate of hydrolysis reaction was investigated. Mathematical model for olive oil hydrolysis was developed including the effect of mixing speed and the interfacial contacting area. To verify the model that was obtained, Olive oil/gum Arabic emulsions were used to perform further experimental analysis. Results confirms that there is a good agreement between the model derived mathematically and results from hydrolysis reactions experiments. The non-linear fitting using Mathematica software was found to more

accurate than applying Hanes-Woolf linear fitting for the experimental data obtained.

3.1 Introduction

The hydrolysis of complex chain oils and its reactions are getting significant attention in recent years. Glycerols and fatty acids produced from oils degradation are very important and had been used in many applications worldwide during the past decades. Recently, the hydrolysis of crude oils, such as olive oil, into free fatty acids and glycerols with high conversion (99%) had been restricted by the applications of high pressure, high temperature, and the very long reaction times [55]. These extreme operating conditions are expensive and in most cases produces dark fatty acids as a result of side polymerization and formation of undesired by-products [55]. In addition, challenges like treatment and purification of the final product using distillation complicates the process and add further energy considerations [55]. The main trend in research these days is to develop an efficient, handy and low cost processes and with optimal operating conditions to produce the desired clean fatty acids.

Recently, lipase has got significant attention and has been applied in many industries as an efficient energy saving biocatalyst. However, the prediction of the kinetics and the reaction behavior of this enzyme needs more investigation. Lipase is a soluble enzyme hence it is enzymatically active in water and organic solvents and because of that, it works at the interface between the oil phase and the aqueous phase which mainly contains the enzyme [56]. Hence, the contacting area between the soluble lipase and the oil phase affects the rate of reaction. This interfacial area

is influenced severely by the substrate concentration and mixing speed. A previous study conducted by Al-Zuhair et al. (2003), on the kinetics of palm oil hydrolysis by lipase assumed that the interfacial contacting area between the enzyme and the substrate remains constant even when changing the agitation and mixing speed [56]. This assumption can be valid for the very high substrate to enzyme concentrations or when good organic solvent such as hexane is used. However, interfacial contacting area has a significant effect on the lipolysis reaction. Since according to Tsai et al. (1991), contacting area between the enzyme and the substrate affected directly by mixing speed and subsequently affects the rate of reaction [57]. Moreover, the use of an organic solvent in the hydrolysis reaction is not an efficient choice because lipase is insoluble in these kind of materials since it would not be absorbed to organic phases and suspended as particles [58]. Furthermore, some applications such as enzymatic waste water treatment add more sophistications and complexity to the application of lipolysis degradation of oils. Not only for the fact that it includes inorganic solvents like water which apparently will affect the solubility of lipase and interfacial contacting area, but also the variety of process designs which includes different mixing and agitation speeds add more concerns about the validity of models that does not consider the contacting area as an important factor [56, 59].

Substrate concentration is one of the key factors that affect the oil hydrolysis reaction. The rate of reaction increases with the increase of concentration. But with high substrate loading the effect is reversed, hence the enzyme active cites will be blocked by the substrates itself. This phenomena is known as substrate inhibition, which con-

sidered almost universal phenomena that restricts the applications and beneficial use of enzymes [60].

The objective of this study was to focus on the effect of the interfacial contacting area, mixing and agitation speed, operating temperature, pH, enzyme loading, and substrate concentration on the rate of reaction and olive oil hydrolysis kinetics. In order to study the effect of interfacial area and the contacting time on the enzyme activity, one has to determine the reaction rate and the amount of fatty acids liberated form the hydrolysis of specified amount of oil using the lipase. It is common and well known procedure addressed by lipase assays protocols in many references [61, 62]. Produced fatty acids form the hydrolysis reaction was determined by direct titration of the liberated free fatty acids with a certain base [61]. Method mentioned above is an easy procedure and have an acceptable accuracy for the characterization of enzymes specially lipases and esterases. Because of the many industrial applications involving lipase [63], investigating and studying its kinetics and optimum operating conditions is a great concern. Subsequently, applications like esterification, transesterification, synthesis of peptides, and region selective acylation of menthols and glycols could be handy and available [63, 64]. Glycerine produced from esterification reacy5tions is used in cosmetic applications, pharmaceuticals, and animal food industries. Besides that, long chain fatty acids such as tripalmitin and triolein are the main products of the hydrolysis of emulsified esters using lipase [34, 65]. These liberated fatty acids are very important and could be used as raw biodiesel fuels [66, 67].

3.2 The kinetics model

The mechanism proposed here of the olive oil lipolysis reaction into simpler fatty acids is similar to that of Alzuhair et al. (2003), and Tsai and Chang models referred to the literature [56, 68]. The enzyme is absorbed in the water interface to produce the penetrated enzyme, E^*. The absorption of lipase is assumed to be proportional to the free specific area, a, and the free enzyme concentration, E. The enzyme-substrate complex, E^*S, is assumed to be the product of the interaction of the substrate, S, with the free enzyme cites [56]. The generated product, P^*, is produced from the complex, E^*S, at the interface and the enzyme, E^*, is reproduced again hence it will not be consumed in the reaction. After this step, the product, P^*, is desorbed from the interface to the bulk phase as final product, P. The proposed mechanism is illustrated in eq:1eq:3:

$$E + a \rightleftharpoons ES \tag{3.1}$$

$$E^* + S \rightleftharpoons E^*S \tag{3.2}$$

$$E^*S \rightarrow E^* + P^* \tag{3.3}$$

As proposed by Tsai and Chang, the quasi-steady state assumption for the adsorbed enzyme and the enzyme/substrate complex, and a proportional relation between the interfacial product, P^*, and the final desorbed product, P, [68]. Also desorption of the product, P^*, to the bulk assumed to be very fast and it is concentration is too low compared to the final product, P. All assumptions stated above were applied to the

model, hence, eq:1eq:3 could be rewritten as:

$$k_p E \cdot a - (k_d + k_1 + k_{cat}) E^* S = 0 \tag{3.4}$$

$$k_1 E^* \cdot S - (k_{-1} + k_{cat}) E^* S = 0 \tag{3.5}$$

$$a_t = a + A_m (E^* + E^* S) a_t \tag{3.6}$$

$$E_t = E + a_t (E^* + E^* S) \tag{3.7}$$

where as, $P* = CP/a_t$ as reported by Al-Zuhair et al. (2003) [56]. By applying the assumption stated previously, the rate of reaction for the formation of the product, v would be:

$$v = \frac{dP}{dt} = \frac{a_t}{C} \cdot \frac{dP^*}{dt} = \frac{a_t}{C}\ k_{cat}\ E^* S \tag{3.8}$$

eq:4eq:8 should be solved simultaneously to get the final expression of the rate of reaction. Similar derivation had been studied and investigated in the literature [56, 68, 69], and it is reported that at low enzyme concentrations, eq:1eq:8 could be solved and simplified to the following:

$$v = \frac{k^*_{cat} E_t S}{K_e (\frac{k_d}{k_p a_t^2} + 1) + S} \tag{3.9}$$

where, $K_e = (k_{cat} + k_{-1})/k_1$ and $k^*_{cat} = k_{cat}/C^*$

The simplified expression above is similar to that stated in the literature [56,68,69].

3.3 Materials and methods

3.3.1 Materials

Candida Rugosa Lipase was purchased from Sigma Aldrich Co., US. Purified high quality olive oil was supplied locally from Hail Agricultural Development Co., KSA. Gum Arabic purchased from Sigma Aldrich Co., US. Absolute Ethanol, Hydrochloric Acid, Sodium Hydroxide, potassium and sodium phosphate buffer, phenolphthalein, and other chemicals that has been used were supplied from local companies with laboratory grads. Deionized water was used as regular solvent for making solutions.

3.3.2 Equipment

A small batch reactor with a water bath circulator and a magnet stirrer was used for the hydrolysis reactions. This reactor was used for studying the effect of different reaction parameters on the lipase activity and olive oil hydrolysis reaction rate. Basically, the effect of conditions like reaction time, pH, temperature, mixing speed, substrate concentration, and enzyme loading on the reaction rate and lipase activity was investigated using this reactor. Burette, volumetric pipette, and Erlenmeyer flasks were used to titrate the fatty acids liberated from the hydrolysis reaction catalyzed by lipase.

3.3.3 Reaction rate and enzyme activity measurements

The lipase activity was measured by lipase units (LU), which is defined as the quantity of lipase that can degrade 1 μmol of olive oil per min at the standard reaction conditions recommended by assay protocol (temperature of 37^oC, Olive oil/gum Arabic substrate, agitation speed 200 rpm, and Sodium Phosphate buffer with pH 8.0). Equations 3.10 and 3.11 below shows the calculations of the lipase units and enzyme specific activity [61].

$$Sp_{act} = \frac{v_0(v+b)}{a} \tag{3.10}$$

$$LU = Sp_{act} \times mg(enzyme\ used) \tag{3.11}$$

Where:

- a Added enzyme amount (mg)
- b Added enzyme volume (ml)
- v Reaction volume (ml)
- v_o Initial reaction rate ($\mu mol\ ml^{-1}\ min^{-1}$)

Olive oil hydrolysis by lipase produces free fatty acids and glycerols as shown by figure 3.1. Fatty acids liberated is the key factor for the reaction rate calculations in this study, hence the lipase activity is defined based on the amount of fatty acids produced. Direct titration for the fatty acids resulted from olive oil hydrolysis with diluted NaOH (0.05 N) was a suitable and accurate procedure to calculate the reaction rate and lipase activity. The amount of the base consumed represents the liberated free fatty acids form the lipolysis reaction.

As recommended by the assay protocol [61], the enzymatic reaction was conducted

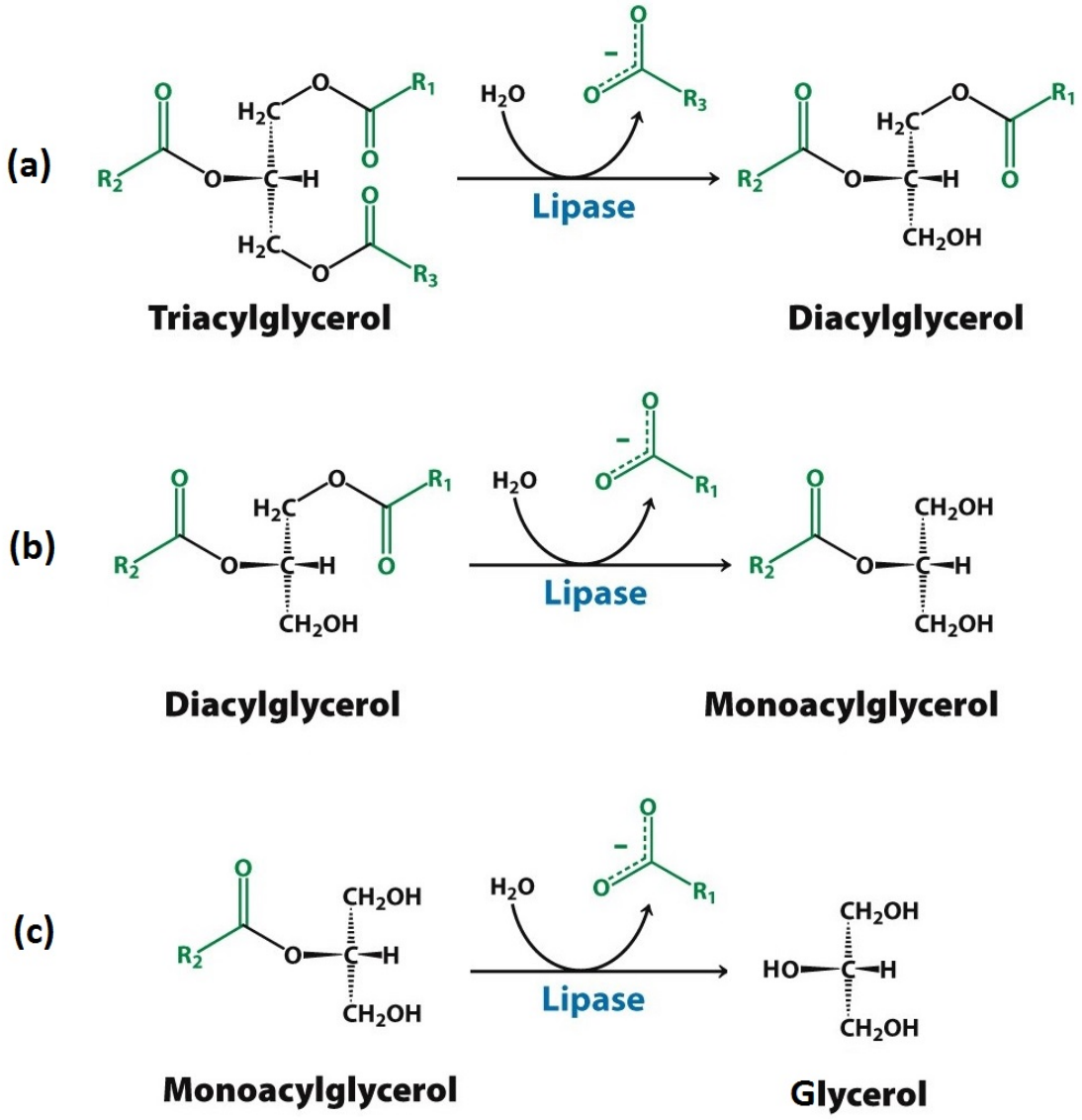

Figure 3.1: Hydrolysis of triglycerides (Oils) into simpler fatty acids and glycerols catalyzed by lipase. (a) Primary hydrolysis of triglycerides into diglycerides, (b) Decomposition of diglycerides into monoglycerols, (c) Final hydrolysis step into free fatty acids and glycerols [70].

using olive oil/gum Arabic emulsion substrate. To prepare the substrate, 10 g from olive oil and gum Arabic were added and mixed in a 400 ml beaker and the volume was brought to 200 ml using 50 mM sodium phosphate buffer, pH 8.0, and was homogenized for 5 min gently using a domestic blender. Then, lipase was added to the emulsion and incubated in 37^oC water bath with continuous stirring. Subsamples was withdrawn at regular intervals and quenched immediately using ethanol to break the lipolysis reaction. By titrating the resulted mixture with NaOH (0.05 N) and taking a blank sample before for calculations, the enzyme activity was determined accurately [61].

Based on the recipe of olive oil/Arabic gum emulsion mentioned above and by adding lipase, the hydrolysis reaction initiated. Typically, 10 ml of ethyl alcohol placed in Erlenmeyer flasks with 25 ml volume and 3 droplets of $1\%wt$ phenolphthalein indicator was added in each to prepare a quenching cocktail in order to suppress the reactivity of sub-samples that would be taken next.

A 50 ml of olive oil-gum Arabic substrate had been pre-incubated for 15 minutes in water bath with 37^0C and a magnet stirring. After the incubation, 50 mg of lipase was added to start the hydrolysis to the emulsion substrate. Using stirred reactor shown in figure 3.2, temperature was kept constant at 37^0C and the timer was started while continuing stirring. At predetermined reaction periods, constant amounts of 5 ml had been removed as sub-samples from the reactor and transferred to an isolated flask which contained the titration cocktail prepared previously. Immediately, the amount removed was swirled and quenched in ethanol to end the reaction. As a

preparatory step for the titration process, the quenched samples was turbid and had been kept for 2 hr at room temperature. The flasks content was titrated using 0.05 M NaOH till a light purple color showed. A plot for the free fatty acids concentration (mol/ml) versus time (min) was generated and the determined slope indicated the initial velocity (v_0).

3.3.4 Lipase loading, temperature, pH, and mixing speed

Table 3.1: Basic Reaction Conditions

Time (min)	Temperature (^oC)	Enzyme loading (mg)	pH	Mixing speed (rpm)
00	20	0.0	6.0	200
05	25	0.1	6.5	500
10	30	0.2	7.0	800
15	35	0.3	7.5	1000
20	40	0.4	8.0	1300
25	45	0.5	8.5	1500
30	50	0.6	9.0	-
35	55	0.7	-	-
40	60	0.8	-	-
45	-	0.9	-	-
50	-	1.0	-	-
55	-	-	-	-

The effect of enzyme loading on the reaction rate was studied using the same procedure stated previously. By fixing the reaction time to 30 min and changing the amount of enzyme from 0.1 mg/ml (lipase/substrate) to 1 mg/ml, the effect of the lipase concentration on the reaction rate was determined. Reaction temperature is another important factor for studying an enzymatic reaction kinetics. Keeping

other parameters fixed, the reaction temperature was changed from 20 to 60^oC and relevant effects on the reaction were clearly observed. Hence, the activation energy was investigated as the temperature increased and plotted to describe the trend of the lipase thermal characteristics and its related effect on the reaction rate. To study the effect of pH, the substrate buffer pH was changed from 4.0 to 9.0 under the same standard conditions and the reaction time was kept at 30 min. Lipase is very sensitive to the change on hydrogen ions. Hence, obvious effect was observed and pH against enzyme activity plots were generated.

3.3.5 Surface area and oils drop size effect

Many methods had been developed for predicting the interfacial area in oil water mixtures [69, 71–73]. The famous Calderbank Model (Equation 3.12) for the area determination was modified by Albasi et al.(1999) and later on by Al-Zuhair et al.(2003).

Calderbank Model:

$$a_t = \frac{6\phi}{D_0} = \frac{\alpha\omega^m\phi T^k}{1+n\phi} \tag{3.12}$$

Where:

a_t	Specific total interfacial area (m^{-1})
α	Constant defined by Eq. 3.12
ϕ	Oil volume fraction in the emulsion
ω	Mixing speed (rpm)
T	Temperature (^oK)
$k,\ m,\ and\ n$	Constants defined by Eq. 3.12

Albasi et al. (1999) modified the model and investigated the effect of increasing

the oil fraction, ϕ, on the accuracy of the results. Their proposed model (Equation 3.13) can predict up to 60% of the oil fraction. Later on, Al-Zuhair [56] did more modification on this model involving the effect of temperature on the interfacial area and drop size distribution (Equation 3.14).

$$A = 2.05 \cdot \omega^{0.745} \phi^{-1.269} \tag{3.13}$$

$$a_t = \frac{0.024 \cdot \omega^{0.6} \phi T^{1.7}}{1 + 3\phi} \tag{3.14}$$

Figure 3.2 shows stirred reactor that used to study the effect of mixing speed and oil drop size on lipase activity. The reactor consists of shell a hot water jacket to control temperature and has an impeller with a motor to manage the mixing speed. The last model, which was modified by Alzuhair et al. (2003) equation 3.14, had been used to determine the drop sizes and droplet distribution for the oil water mixture in order to study its effect on the enzyme activity.

3.4 Results and discussion

3.4.1 Lipase activity

To check the enzyme activity, method described above is section 3.3.3 was used. As shown in figure 3.3, the amount of fatty acids liberated by lipase is increasing rapidly as the reaction time increases. Since, initially the enzyme starts to be absorbed in to water phase and consequently starts to activate. Thus, as the time goes more sub-

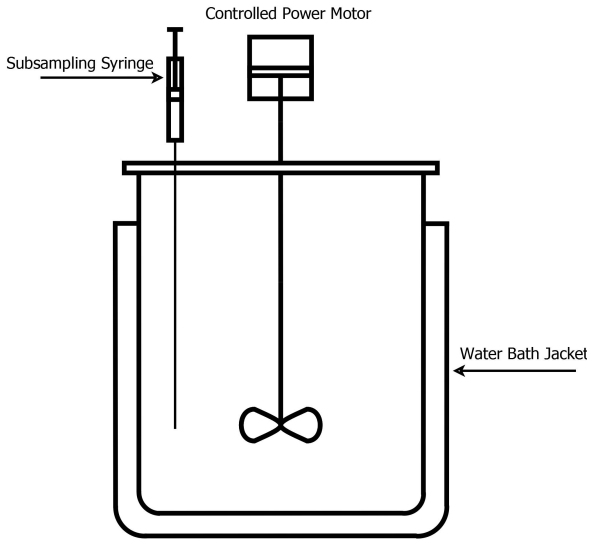

Figure 3.2: The experimental setup for the lipolysis of the olive oil emulsion

strate starts to react on the enzyme surface. For lipase concentration of 0.2 mg/ml (enzyme/substrate), results shown in figure 3.3, indicate that the specific activity, sp_{act}, which is defined by equation 3.10 was found to be 2.57 $\mu mol/(mg{\times}min)$ according to the initial rate of reaction (v_0) obtained from the linear fitting for the data.

Enzyme units, LU, was calculated and found to be 25.67 which is very close to what Krakowiak et al. (2003) got and even better since they got an activity around 22 LU when they studied the lipase immobilization on chitosan polyphosphate [74]. This result indicates that the used enzyme is active and efficiently degraded the triglyceride into fatty acids.

3.4.2 Effect of enzyme loading on reaction rate

As shown in figure 3.4, the effect of enzyme concentration on the lipolysis reaction show that for the stated conditions, the reaction mixture reached a saturation point

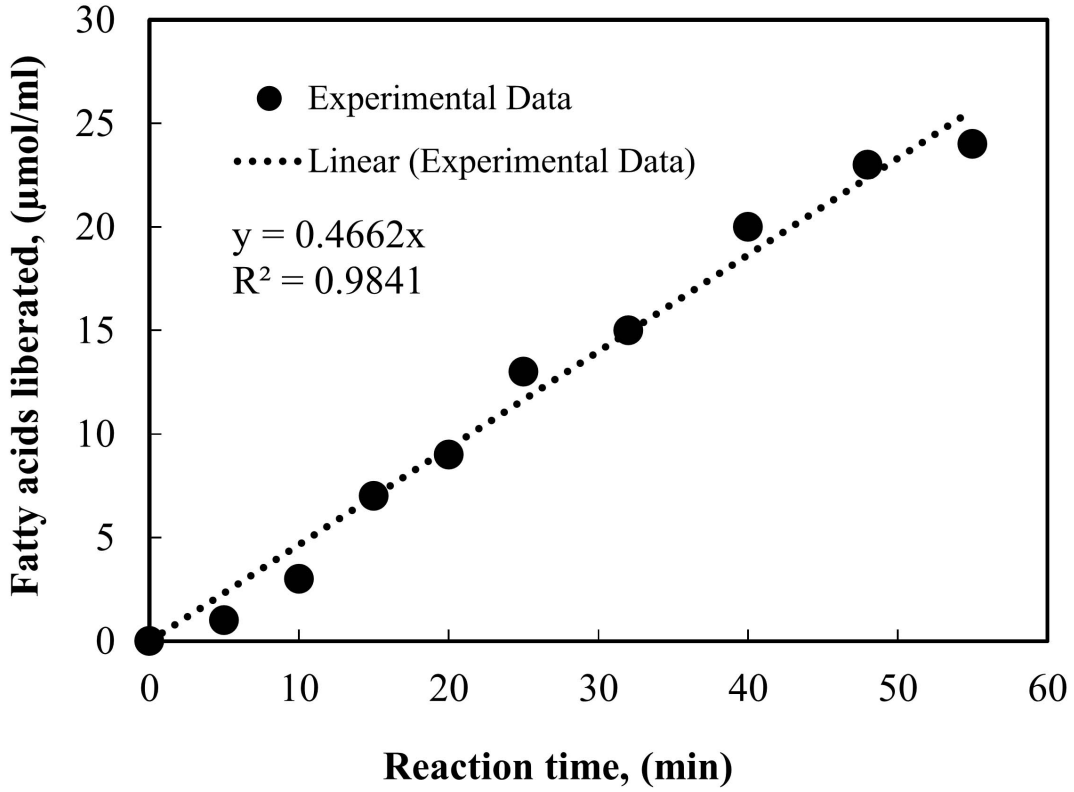

Figure 3.3: Initial reaction rate (velocity) calculations using linear fitting, ($[E] = 2\ g/L$ (lipase/substrate), $\omega = 200\ rpm$, $T = 37\ ^oC$, $pH = 8.0$) [75].

at 0.7 mg/ml. After this point, adding more enzymes does not affect the reaction rate. This is due to the saturation of the substrate solution by the enzymes, thus, no more substrates left to get involved with the extra free lipase. Alzuhair et al. (2003), they studied Candida Rogusa lipase and they found that the rate of palm oil hydrolysis levelled with a constant value after 0.1 mg/ml [56]. This poor results that they got compared to ours is due to the high temperature that they used (45^oC) which is slightly above the optimum range as reported by many other studies [76]. However, our findings and results are still comparable to what they observed.

In another study done by Saktaweewong et al. (2011), they found the maximum lipase loading to be about 0.26 mg/ml, this is close to what we observed because they nearly obtained their results under similar conditions [77]. The main reason behind the inhibition of the enzyme after this critical point is the formation of multilayer at the interface where the reaction takes place. Below this concentration a monolayer was dominating the adsorption at the interface and the rate of reaction was linearly increasing with the lipase concentration as clear from figure 3.4. Increasing the enzyme loading increases the layers rounding the substrate and, therefore, decreases the interaction between the enzyme and the substrate bulk. To avoid this phenomenon, either we have to decrease the amount of enzyme added or increase the mixing speed to allow more agitation and refresh the surface od the enzymes. Even this last one has its limitations [57].

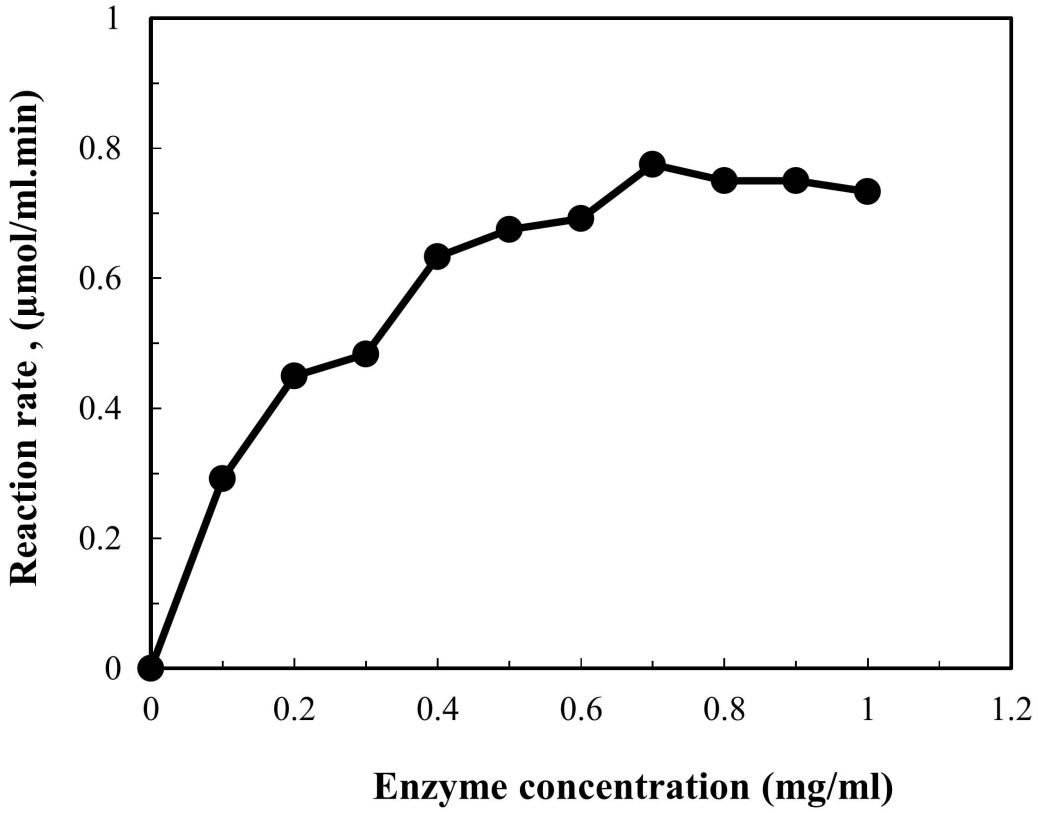

Figure 3.4: Enzyme concentration effect on the reaction rate and activity, ($t = 30\ min,\ \omega = 200rpm,\ T = 37^oC,\ pH = 8$)

3.4.3 Effect of pH on the lipolysis reaction

Figure 3.5 shows the effect of pH on the lipase activity. It shows that the enzyme activity remains high at a pH range from 7.5 to 8.5 while it starts to decrease at lower and higher values. These results are identically confirms previous work done by many in the literature [34, 44, 74]. Gupta et al. (2011) reported that, optimum pH of 8 when they characterized Candida Rogusa lipase after immobilization on membranes which is typical to what we obtained at nearly similar conditions. The clear decrease of enzyme activity below and above this optimum range is because of the reversible reaction that occurred due to the ionization and deionization of basic or acidic groups in the active cites of the lipase [78].

3.4.4 Substrate concentration effect and kinetic modeling

The substrate concentration has great effect on the lipolysis reaction. Figure 3.6 shows that the reaction rate is increasing with the increase of the substrate concentration in the emulsion. This is due to the high enzyme capacity compared to the reacted oil at the beginning of the reaction. However, from figure 3.8 and considering the density of olive oil to be 920 Kg/m^3, when the oil fraction reaches a range of 30 to 40%, the reaction rate levelled at constant point. A saturation point was observed and the rate of reaction remains fixed at 17 $mol/(L\ min)$. Thus, after this critical concentration the enzyme active cites can not absorb more substrate. This is similar to what Alzuhair et al. reported when they was studying the hydrolysis of palm oil by lipase in nearly close conditions [56]. Alzuhair et al. also reported that, after 43%

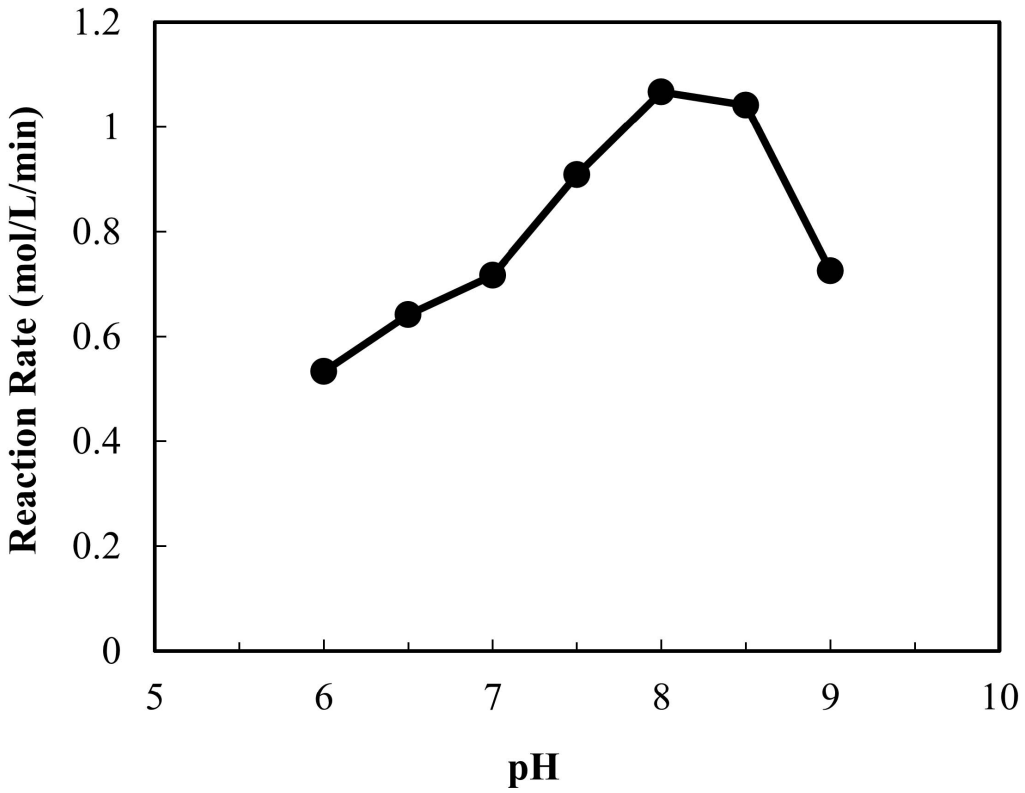

Figure 3.5: Effect of pH on the reaction rate and enzyme activity, ($[E] = 1g/L,\ t = 30min,\ \omega = 200rpm,\ T = 37^oC$,)

of oil fraction the reaction rate decreases and the substrate causes inhibition to the enzyme [56]. In our case, increasing the olive oil fraction up to 55% did not affect the rate of reaction that much. It is clear form the results obtained that the substrate has relatively low affinity to the enzyme, since K_m for the enzyme was found to be almost about 0.95 g/ml which is quit high as compared to the results obtained in the literature [79, 80].

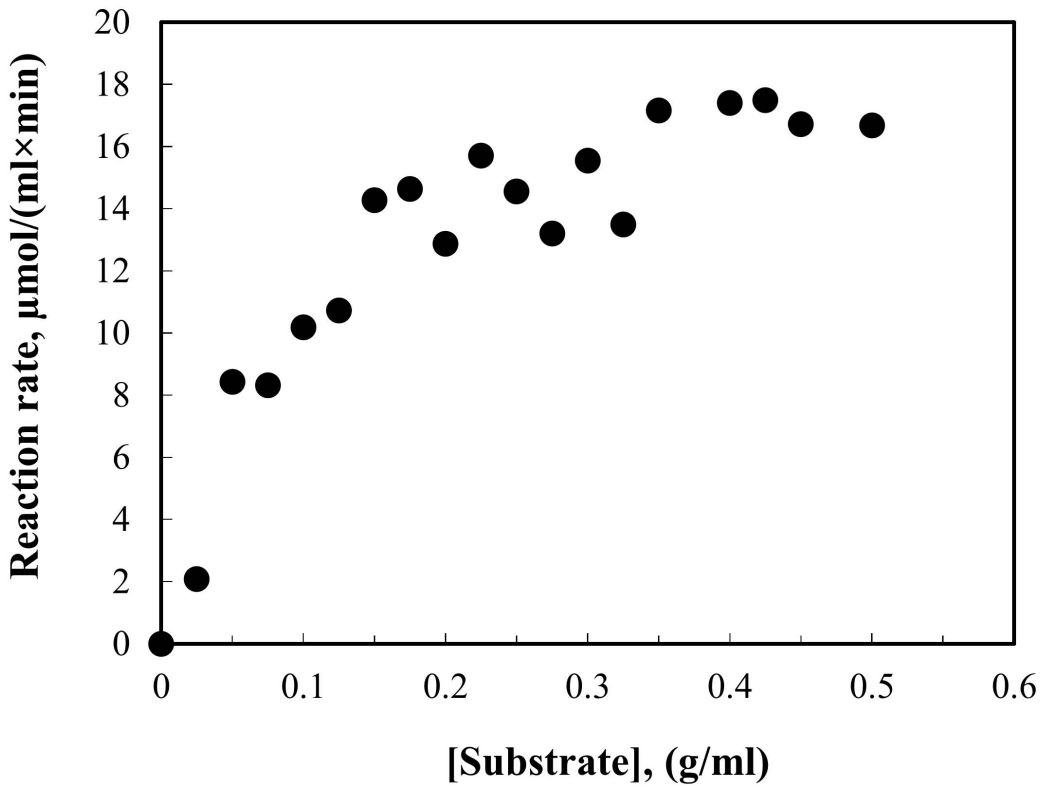

Figure 3.6: shows the reaction rate and the effect of the substrate concentration

3.4.5 Experimental data fitting and constants evaluation

Many studies recommend Hanes-Woolf model to fit the experimental data to Michaelis-Menton Model [81, 82]. Results in figure 3.6 were fitted using Hanes-Woolf method of linear fitting as shown in figure 3.7. The plot of $\frac{[S]}{v}$ $versus$ $[S]$ gives accurate results as recommended by Nimmo et al. (1975) [83]. With this fitting, the estimated values for $V_{max} = 22.727\ mol/(mg\ enzyme \times min)$ And $K_m\ is = 0.1364 g/ml$.

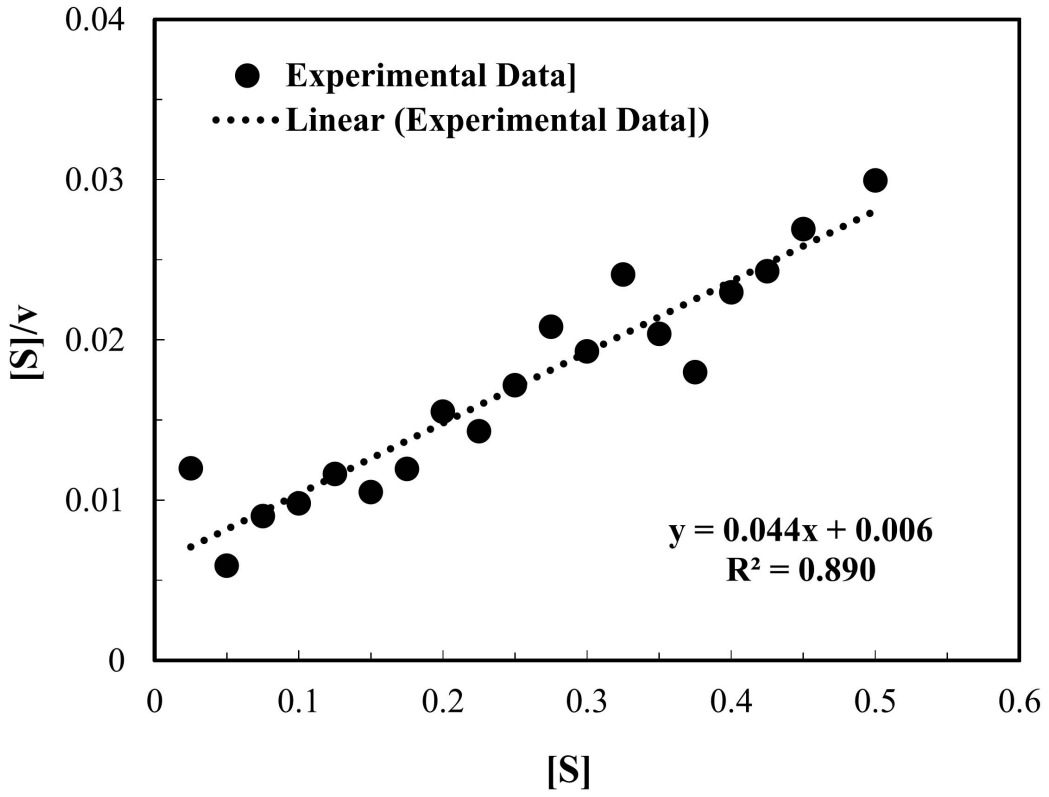

Figure 3.7: Hanes-Woolf plot for experimental data

A nonlinear fitting for the data to the proposed model was directly obtained using Mathematica software. As shown in figure 3.8, high correlation coefficient with R^2 equals to 0.992 obtained and the estimated values for V_{max} was about 20.514 $g/(m^3 min)$

which is very close to Hanes-Woolf fitting. The K_m which represent the substrate affinity is found to be about 0.0975 g/ml which confirms the results obtained by Al-Zuhair et al. [56]. Hence, they found the value of K_m about 0.076 g/ml [56]. This slight difference is because they used higher mixing speed (800 rpm) when studying palm oil hydrolysis, since increasing the stirring and mixing decreases the affinity to the substrate concentration [56, 72].

$$v = \frac{20.514[S]}{0.097 + [S]} \tag{3.15}$$

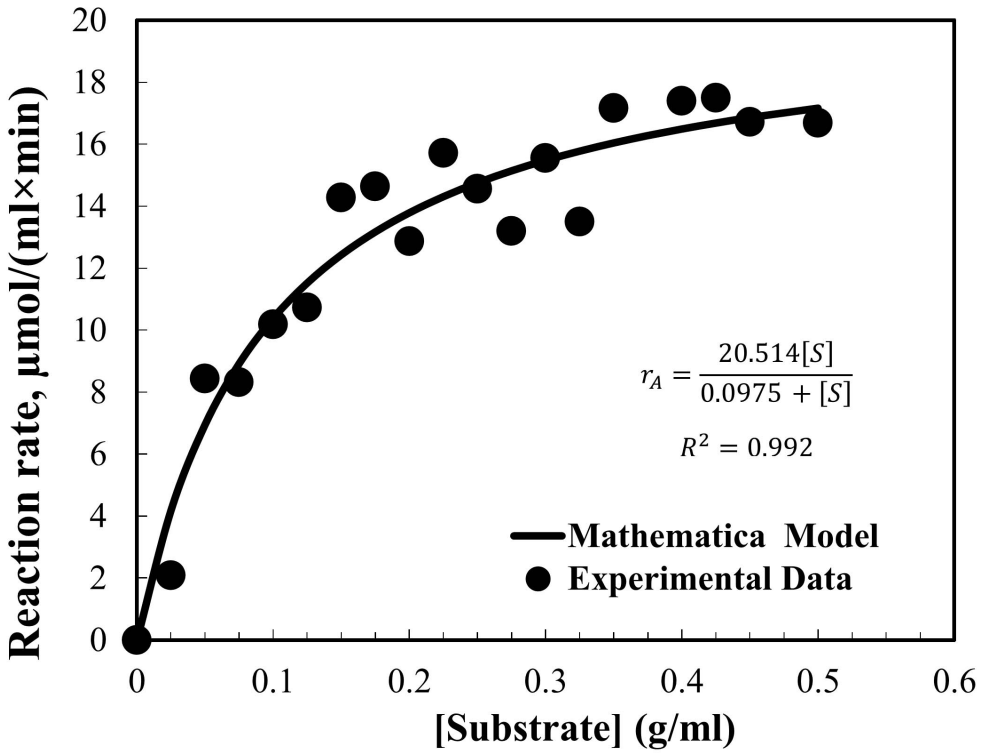

Figure 3.8: Nonlinear fitting using *Mathematica*© software for the experimental data to Michaelis-Menten Model

3.4.6 Effect of mixing speed on the rate of reaction

Figure 3.9 shows the effect of mixing speed on the interfacial area between the enzyme and the substrate. It is well-known that increasing mixing and agitation leads to smaller oil droplets dispersion and subsequently it affects the rate of reaction. Equation (3.14) was used to estimate the specific area for olive oil/water emulsion. As clear from figure 3.9, the specific interfacial area increases proportionally with mixing speed. Subsequently, figure 3.10 shows that the rate of reaction also increases as the mixing speed increases. However, after 900 rpm no more effect occurs on the reaction rate. At this point the enzymes active cites are fully saturated and no more oils could be added. Many studies reported similar behavior for oil hydrolysis using lipase hence the specific interfacial area has no effect at very high mixing speeds [56, 72, 84]. Applying equation 3.14 the interfacial area could be estimated and a new coefficients for equation 3.9 could be obtained.

3.4.7 Activation energy and temperature effect

As shown in figure 3.11, the reaction temperature had significant effect on the rate of reaction. Increasing the temperature increased the initial reaction rate rapidly in the range below 40^oC due to primely two phenomena: (1) Because of the nature of lipase of being an enzyme that affected is severely by temperature, therefore, its activity maintained hight inside this range; (2) While the temperature is increasing the interfacial area increases due to the thermal effect and mixing enhancement. similar results had been obtained by Al-Zuhair et al. when they studied the effect of tem-

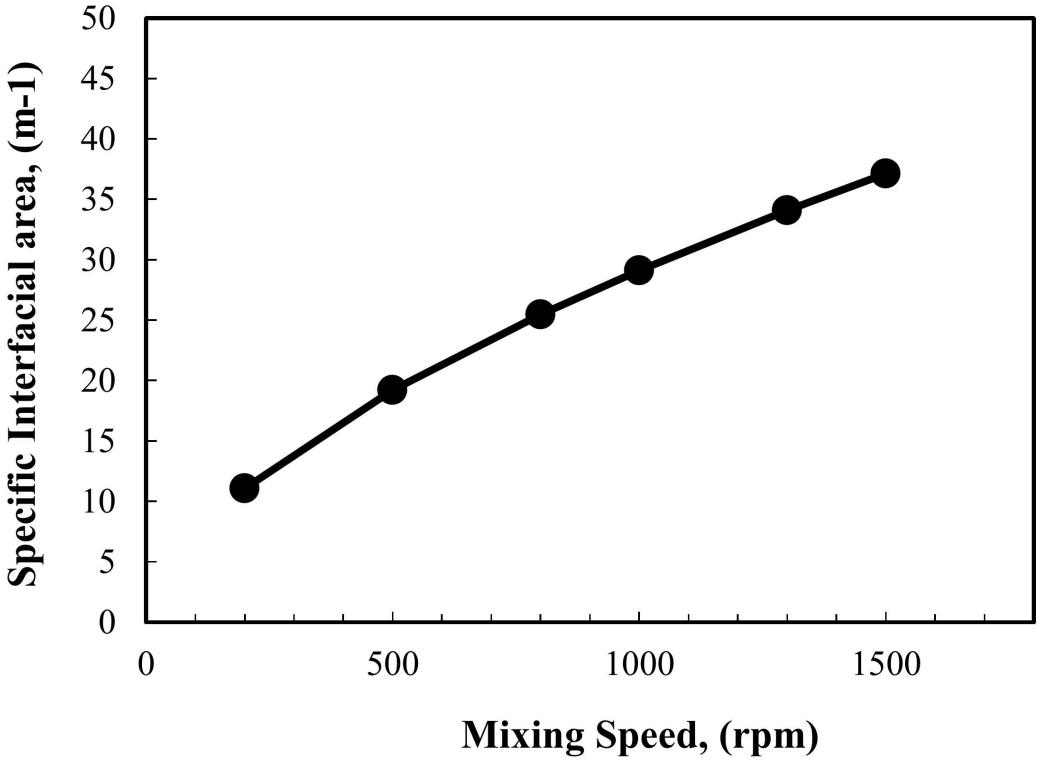

Figure 3.9: The effect of the mixing speed on specific interfacial area, ($\phi = 0.05$, $T = 37^oC$,)

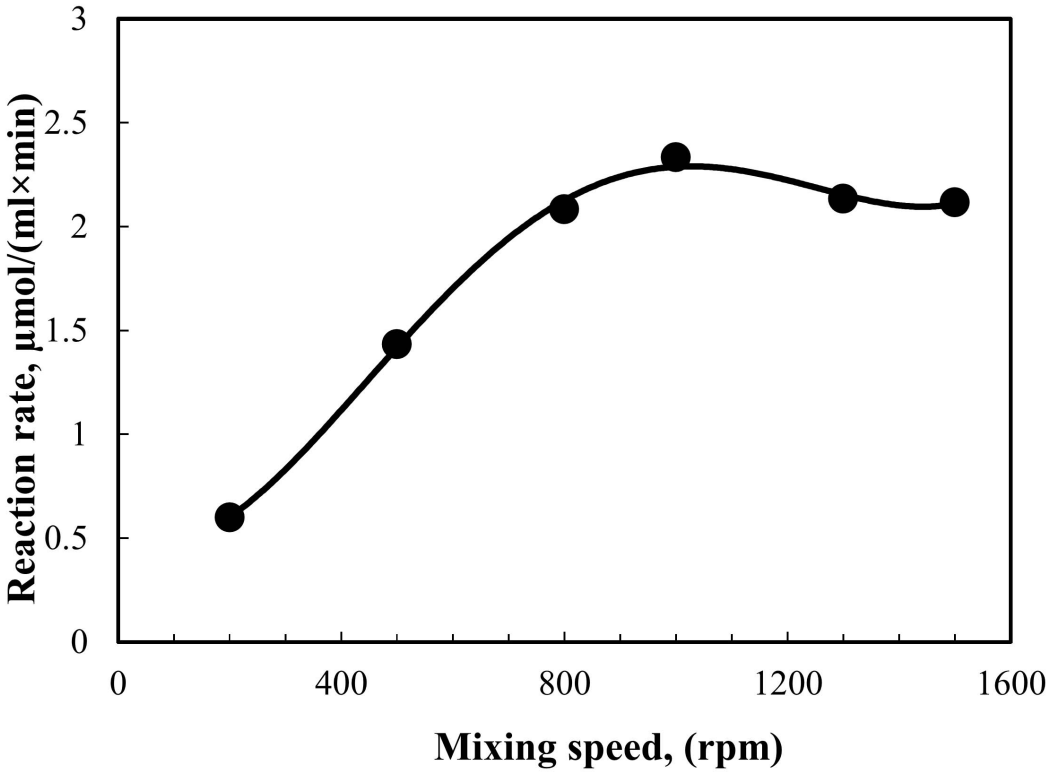

Figure 3.10: The effect of the mixing speed on the reaction rate and enzyme activity, ($[E] = 1g/L,\ t = 30min,\ pH = 8,\ T = 37^oC$,)

perature on the lipolysis of palm oil [56]. Above 40^oC, a significant decrease on the reaction rate has been noticed. Mainly, this is due to the deactivation (denaturation) of the enzyme, hence it is affected by the high temperatures increase and starts to decompose in that ranges [85]. A sharp rapid decrease in the activity following the previous stage because of the blockage of the interface between the active enzymes and the substrate by denatured enzymes, which add more resistances to the hydrolysis reaction to occur.

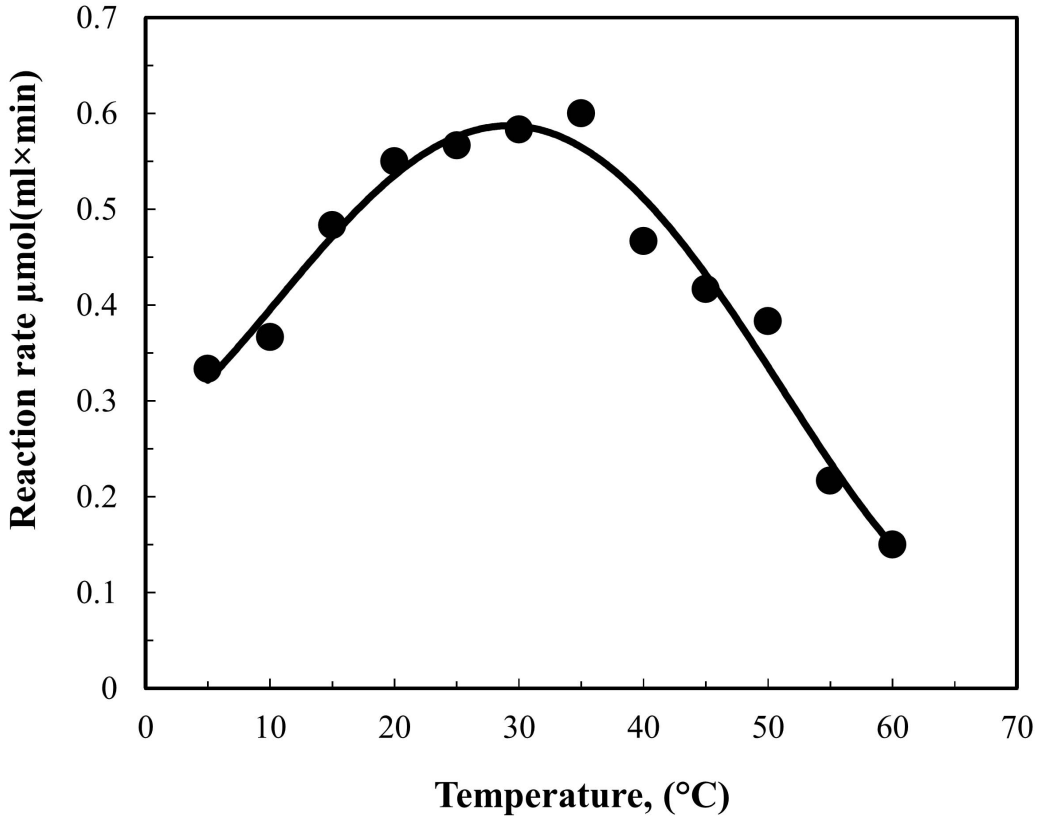

Figure 3.11: The effect of the temperature on the reaction rate and enzyme activity, ($[E] = 1\ g/L, t = 30\ min, \omega = 200\ rpm, pH = 8$)

The reaction constant k_{cat} had been estimated from figure 3.11 and the effect of

the temperature and the activation energy on the rate of reaction has been stated using the following relation:

$$k^*_{cat} = k_o exp(\frac{E_{act}}{RT}) \tag{3.16}$$

From figure 3.12 shown below, values for k_o and E_{act} were determined and it was found that the activation energy was around 4.32 $Kcal/gmol$, which is quit acceptable compared to what was reported for the enzymatic reactions [86]. Hence, Al-Zuhair et al. reported a value of 1.2 $Kcal/gmol$ for the hydrolysis of the palm oil using batch stirring reactor [56]. Kim and Chung reported 7.0 $Kcal/gmaol$ activation energy when studied the hydrolysis of palm oil in reversed micelles system [56]. However, the most close results stated in the literature to the one here is 5.3 $Kcal/gmol$ reported by Sémériva and Densulle (1979) when they studied the hydrolysis reaction for oil/ water emulsions. [87].

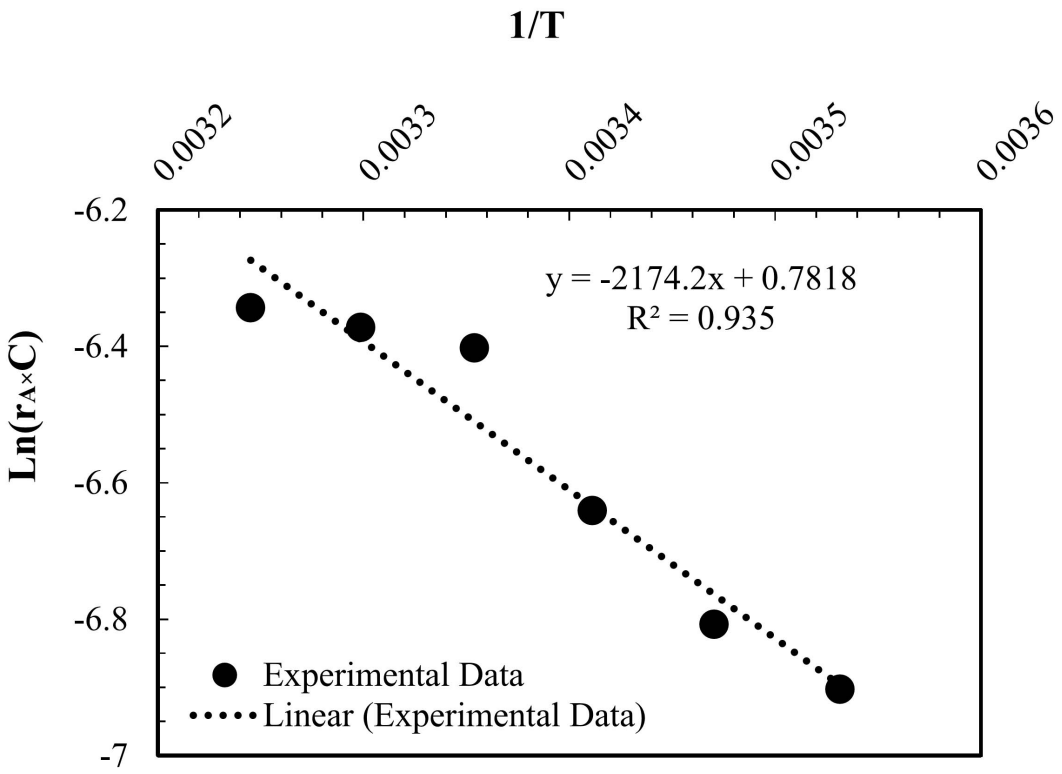

Figure 3.12: Shows the activation energy for the lipolysis reaction

3.5 Conclusion

A detailed kinetic model for the hydrolysis of olive oil by lipase enzyme was obtained. Reaction temperature, mixing speed, pH, and substrate concentration were included in this study. It was found that the optimum conditions for the hydrolysis of olive oil were 35^oC, pH of 8, and 0.7 g (enzyme)/ml (emulsion). Olive oil emulsions hydrolysis was performed to verify the proposed model experimentally. It had been shown that lipase is very sensitive to mixing speed in the range between 200 and 1000 rpm, since it affects the interaction contacting area between the enzyme and the substrate. The proposed model had a very good agreement with the experimental data obtained. Substrate affinity was found to be 0.0975 g/ml and the activation energy for the hydrolysis reaction was 4.32 $Kcal/gmol$ which agrees with what stated in the literature. The coefficients estimated using the experimental data could be used to predict the behaviour of similar hydrolysis reactions in similar conditions.

CHAPTER 4

SYNTHESIS AND FUNCTIONALIZATION OF MULTI WALL CARBON NANOTUBES

4.1 Introduction

Recently, focus and interest has been made to carbon nano-structured materials. Since the discovery of fullerenes, carbon nanofibers (CNF), and carbon nanotubes (CNT), these type of materials became interesting and had great commercial and industrial importance [35]. CNT and CNF became very important materials to the recent nanotechnology revolution. Because of their mechanical, electronic, optical, and chemical properties, it opened the way to many future research and applications. Remarkably, mentioned properties can be observed even in one single nanofiber or nanotube [37,88].

During the last two decades, researches showed that applications like electronics (transistors, capacitors), energy storage, nano-mechanical devices, and nano-composite materials are perspective applications for carbon structured materials.

Back to 1991, Sumio Iijima observed a helical structures carbon materials while he was analyzing carbonaceous materials using Transmittance Electron Microscopy (TEM) [38]. He used arc discharge method for the synthesis and since then this new discovered material named "carbon nanotube (CNT)". CNTs has a diameter nearly between 2.5 nm and 30 nm and its length varying from few nanometers to micrometers. Following this interesting discovery, CNT synthesis and analysis became an area of interest and focus among researchers. Ebbesen and Ajayan (1992), they reported that increasing pressure inside arc discharge chambers remarkably enhanced the yield of CNT production at the graphite cathode. Continuing his work in synthesizing CNTs using arc discharge technique, Iijima and Bethune (1993), they synthesized one nanometer diameter carbon nanotubes [39]. Smalley et al. (1996), figured a new methodology of producing single-wall CNT with extraordinary homogeneous structures and uniform diameters using laser vaporization of graphite. CNT produced using this technique had the property of forming tube bundles aligned together, and they guided Smalley and his group to invent the "rope bundles" [41]. Yacamán et al. (1993), they produced CNT by using chemical vapor deposition (CVD) in their labs which considered significant achievement. By discovering this technique, CVD, the door for synthesizing the CNTs commercially was opened widely around the world because it is an easy and efficient procedure of synthesizing such materials [89].

4.1.1 Structure of Carbon Nanotube

Carbon nanotubes is described as a rolled sheet of graphene into tubular shape which has a hexagonal structure of carbon molecules. So, the shell of the tubular structure consists of benzene-like hexagonal cycles of carbon molecules. The end of the tube is covered by a cape of half dome (half fullerene) shape. As described, CNT has one dimensional structure which gives it the most important and unique physical properties like having a metallic or semi-conducting The chirality of the carbon molecules on the shell side controls the electrical conductivity of the material. Because it is well-known as a large aspect ratio one dimensional material and it is nano-cavities, carbon nanotubes is expected to be used in many different applications. Applications like hydrogen storage as fuel, electrode materials, capacitors and field emitters considered hot areas of implementation and use of such interesting material [90]. Because of all previous properties mentioned above, specially the high surface area, CNTs can work as an excellent matrices to support bio-materials like enzymes. Enzyme immobilization is a virgin area of research when it is coming to the use of nano-materials as supports. Because they enhance the enzyme physical and chemical characteristics, carbon nanotubes are nowadays a key factors when determining the efficiency and reliability of the biocatalysts. For its unique properties, CNTs are in forefront of recent materials proposed to be used in such important application. High surface area, ability of functionalization, efficient mass transfer, severe industrial condition resistance, and effective enzyme loading are the most desired advantages of such enzymes support [91]. Therefore, covalent attachment of enzymes like lipase to functional-

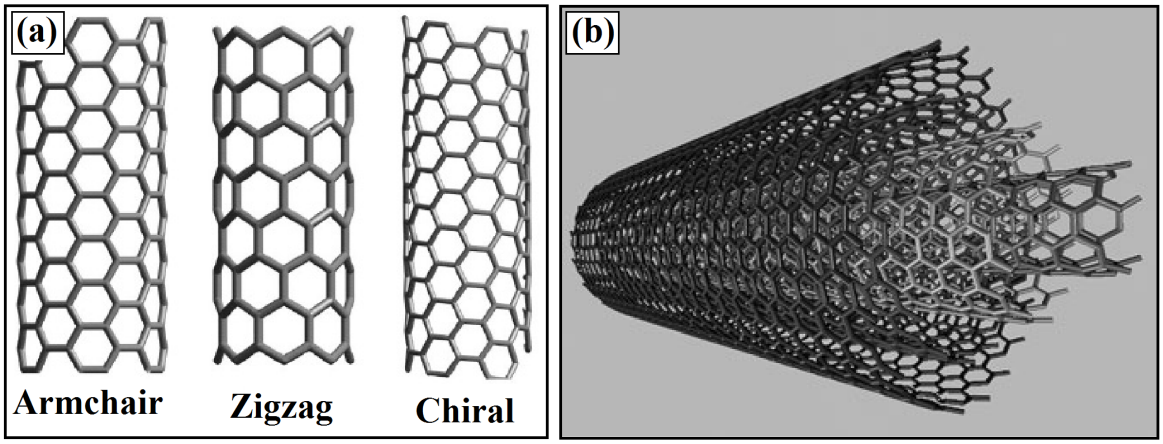

Figure 4.1: Different types of carbon nanotubes, (a) single wall with different structures armchair, zigzag, and chiral , and (b) multi-wall carbon nanotubes

ized CNTs became famous technique to enhance their thermal stability and industrial durability recently [10, 92–94].

4.1.2 Production Methods of Carbon Nanotubes

Carbon nanotubes with different types and aspect ratio are produced in various ways these days. However, the most well-known methods used recently are: chemical vapor deposition (CVD), laser ablation, and arc discharge [95]. Although of the many methods and techniques of synthesis, yet looking for cost-effective large scale production and purification methods is major concern for researcher and commercial. Mainly, arc discharge considers two carbon electrodes in which a vapor is created between them in the presence or absence of catalyst to synthesize the nanotubes. In laser ablation technology, highly concentrated beam of laser is imposed to a carbon source which includes feedstock gas like carbon monoxide or methane. Laser ablation technique is well-known by the production of highly pure CNTs but with small amount. On other hand, arc-discharge technique produces large quantities of nanotubes with poor

quality. Chemical vapor deposition considered the most promising technique for the industrial large scale production. This is due to the low growth and reaction temperature, which adds cost advantage, and the high production yields with acceptable purities and thus it is preferred as an efficient synthesis technique [95–97].

4.1.3 Chemical Vapor Deposition (CVD)

Chemical vapor deposition considered a useful method for coating production, fibers, powders and monolithic components. Recently it is considered an important factor in electronics industry, bearings, tools coating, corrosion, and many optical applications. Chemical vapor deposition depends on the principle of solid deposition on pre-heated surface after a vapor phase reaction takes place. It is a sub-class from vapor transfer methods, in which a reacted atoms or molecules or both of them deposits in an atomistic behavior. This method includes many hydrocarbons as a carbon source. Benzene (C_6H_6), acetylene (C_2H_2), Xylene (C_8H_{10}), methane (CH_4), pentane (C_5H_{12}), and carbon monoxide is cracked and deposited over different kind of metallic catalysts such as Fe, Co, and Ni at a temperature range from 500 up to 1200^oC. Historically, CVD had been used as an efficient synthesis process for carbon fibers production [98–100] and nanofiber [45, 101–103], but the use of this method to produce CNT was not investigated. In 1993, Yacaman et al. studied this technique as an excellent method to synthesize carbon nanotubes [89]. Chemical vapor deposition technique depends on the deposition of hydrocarbon atoms on heated catalyst. The hydrocarbon molecules is dissociated by the catalyst forming complete new structure

of nano-material. Figure (4.2) illustrates the chemical vapor deposition reactor that was used in the experiments.

4.2 Materials and methods

4.2.1 Synthesis of carbon nanotubes

A vertical CVD reactor which shown in figure 4.2 was used to produce the required CNTs. This type of reactors gives good quality and quantity of CNTs as reported by AlSaadi et al. [104]. It is very important to have a carbon source (hydrocarbon) to be cracked into free active carbon atoms which considered the nucleus for nano-material initiation. While it is flowing through the quartz tube (reactor vessel), carbon source is heated to high temperatures. This energy is introduced to break down and crack the hydrocarbon molecules into reactive carbon atoms. It is important to explain the floating catalyst technique that was used in this experiments. Mainly in typical CVD reactors, cracked carbon diffuses to the prepared substrate inside the reactor. This substrate is coated with a "metallic" catalyst that controls the nanotubes growth. Ni, Fe or Co are the most well-known metals that catalyze the CNT growth. In our case an "organo-metallic" catalyst was used to control the carbon growth and the structure of the nanotubes. Many metals forms a chemical binding (Sandwich bonds) with organic unsaturated hydrocarbon rings (cyclopentadienyl) forming a material that has both organic and metallic properties well-known as Metallocenes. Ferrocene, Nickelocene, and Vanadocene dichloride are examples of metallocene. This type of materials can

be dissolved in most organic solutions forming a homogeneous organic mixture that contains metals. Ferrocene is one of the efficient organometallic catalysts that is used for the synthesis of CNTs. Para-Xylene was purchased from a local company and used as carbon source for the reaction. Before introducing p-Xylene to the reactor, it was mixed with Ferrocene ($2\%wt$) to form the homogenous mixture that will be vaporized inside the reactor. Ferocene is an organo-metallic catalyst used for CNT growth hence it controls the type of CNTs produced and the aspect ratio. An automated syringe pump as shown in figure 4.2 was used to feed and control the flow rate of the mixture into the quartz-tube inside the reactor. The reaction temperature was set to $850^o\ C$. Hydrogen gas was used, in-excess, as a reducing agent during the reaction to maintain the cracking of the carbon source [105, 106]. It is important to mention that the reaction takes place in an hour after the oven reaches the optimum temperature. According to these conditions, high quality Mw-CNTs is expected to be produced.

4.2.2 Oxidation and acid treatment of the produced Mw-CNTs

For the produced Mw-CNT, each 0.3 g were sonicated in 25 ml of concentrated nitric acid (65 wt%) in a condenser flask as shown in figure4.3. The dispersion was refluxed under magnetic stirring and continuous heating of 150^oC for 48 h. After that, deionized water was used to dilute and wash the treated Mw-CNTs and filtered. The resulting diluted nanotube-acid mixture was then introduced to a centrifugal to separate the nanotubes from the water. Typically, 9000 rpm and about 10 min was

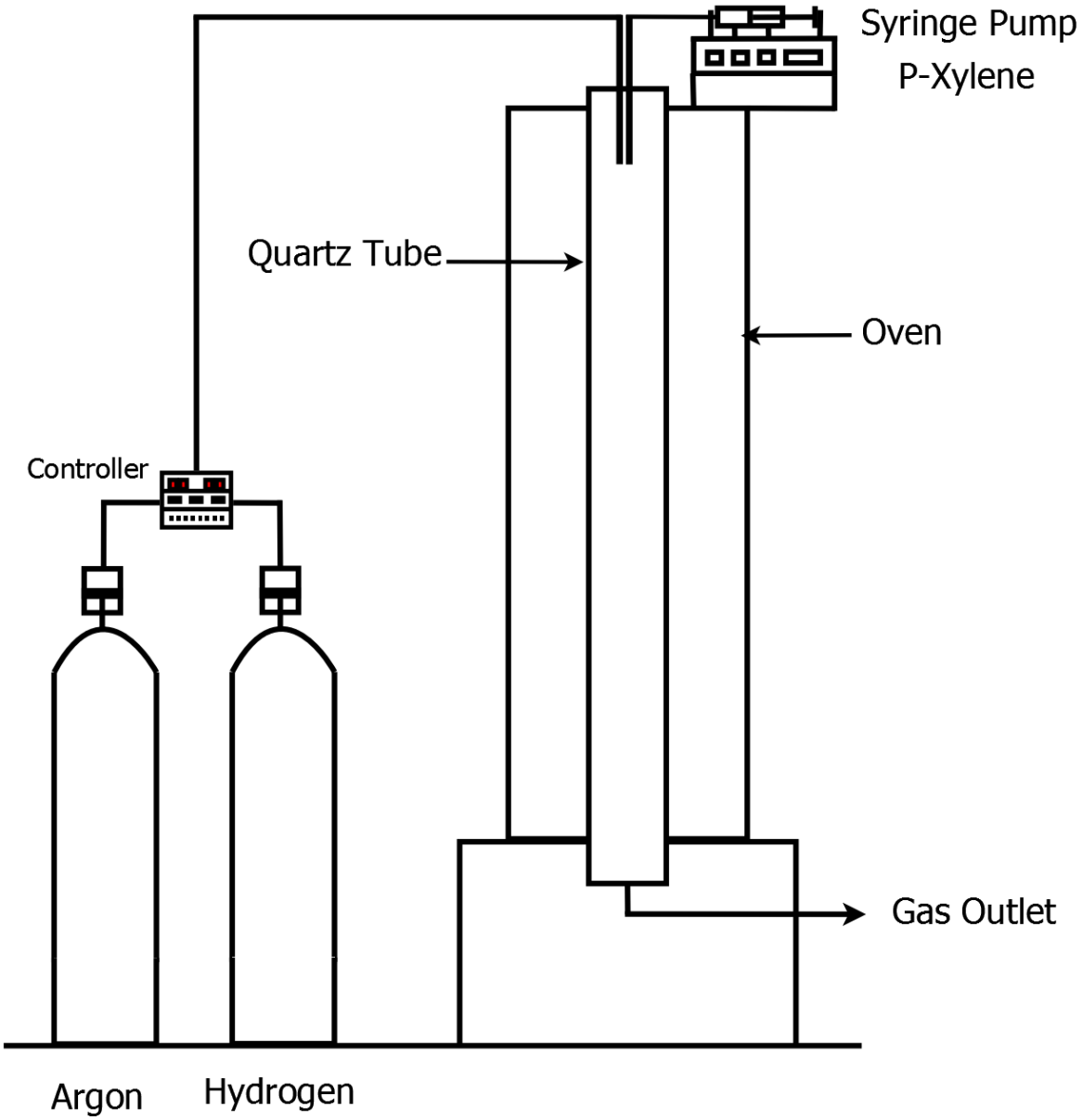

Figure 4.2: Vertical chemical vapor deposition reactor used in this study, $T = 850^{o}C, H_2 = 22.5\ L/hr, Ferrocene = 2\ \%(wt), p-Xylene = 40ml/run, t = 1\ hr$

enough to separate the nanotubes completely from the water. Finally, the nanotubes were rinsed using ethanol up to neutral pH (7) and the samples were dried in vacuum at 40^oC overnight. [107, 108].

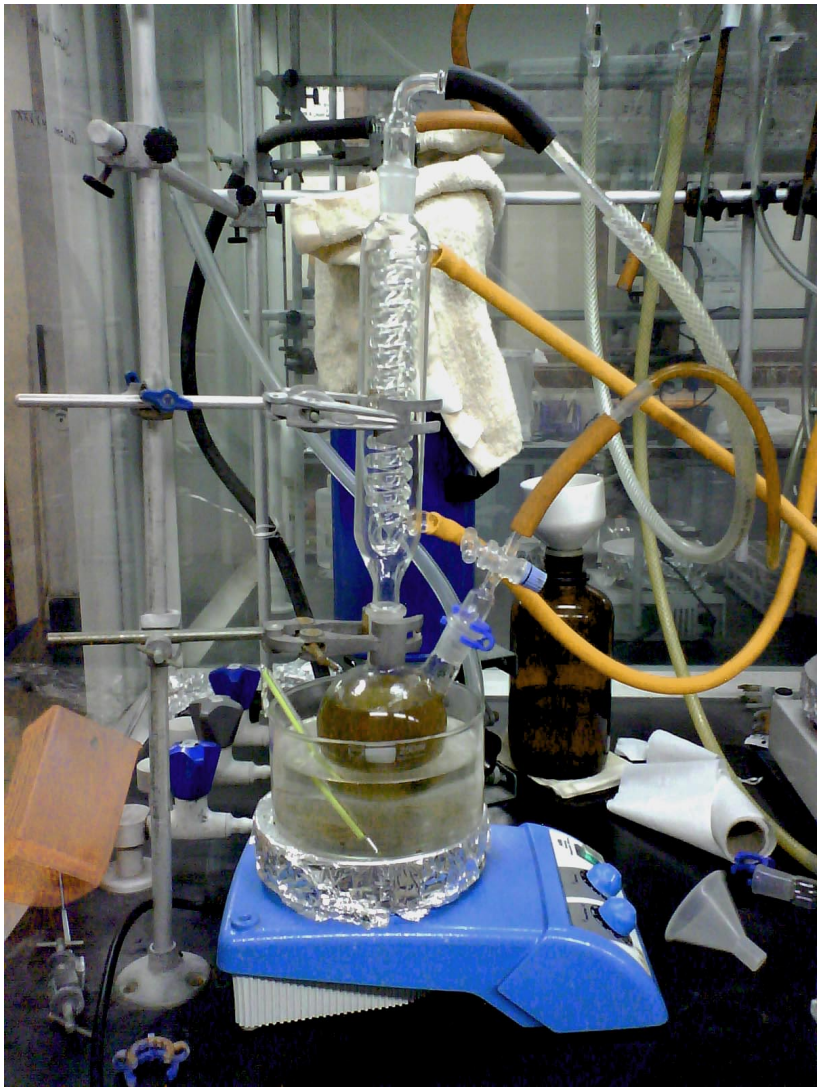

Figure 4.3: Reflux condenser used for the acid treatment of Mw-CNT produced from CVD technique, $T = 150^oC, HNO_3 = 65\%wt, CNT = 0.012g/ml, t = 48hr$

4.2.3 Characterization of the produced Mw-CNTs

Scanning Electron Microscopy (SEM) analysis

Scanning electron microscopy (SEM) was used to analyze and investigate the morphology (aspect ratio) of the produced Mw-CNTs. For its ability and accuracy in scanning the samples surface structure, SEM gives excellent indication for the outer shape and general physical characteristics of the samples. Typically, a very small amount (few μg) of the produced CNTs were prepared on a 12.7 mm pin which is covered by carbon tabs. Gold coating was applied for each sample (5 nm gold layer) before analyzing using the SEM device. Different magnifications and energy levels was used. Samples before and after acid treatment was imaged clearly using different positions.

Thermogravimetric Analysis (TGA)

Samples purity and thermal stability of the produced Mw-CNTs was analyzed using thermogravimetric analysis known as TGA. It was used to study the crystalinity of the produced CNT in order to distinguish the deposited carbons according to their different thermal stability level. This analysis test was performed using a TA Q500 thermogravimetric Instrument which has an EGA furnace installed. Each sample was prepared and 7 mg was taken to the platinum pan inside the instrument furnace. Heating rate was adjusted to be $1^oC/min$ with maximum temperature of 800^oC. The test was performed in air flow of $100ml/min$ through the burning period. Purification of sample was not necessary, since the CVD technique produces Mw-NTs with high purity ranged between 92.5% up to 97.1% [109].The only impurities expected to appear

in the produced Mw-CNTs were an amorphous carbon thin layer on carbon nanotube surface. The presence of encapsulated Fe catalyst particles inside the tubes is probable.

Fourier Transform Infrared Spectrometry Analysis (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a very important analytical tool when coming to the chemical biding and material surface modification. Although it is useful and a direct analytical tool to study the nature of oxidized surfaces, yet there are many experimental issues keeps us from getting IR spectra for carbon materials [110]. It could only be used for carbon material with high oxidized surfaces; else the bands absorption intensity will be very low. A factor that has hindered the advancement of FTIR as a tool for Mw-CNT analysis is the poor infrared transmittance of Mw-CNTs [111]. The use of KBr to dilute the samples which allows more IR to pass throw the sample is one of the well-known techniques used in such cases [112]. Mw-CNTs shows a very strong absorbance due to its dark nature, thus it can not be distinguished from noises of the background during FTIR analysis. Therefore very small concentration of the sample is used usually diluted with potassium bromide. In this study, samples were prepared using potassium bromide and a tiny crystals of Mw-CNTs added. After grinding the CNT into potassium bromide, a manual press was used to make small plates with size fits to FTIR instruments light beam holder. The number scans used varied between $8 - 16$ scans with a wave length range from 500 cm^{-1} to 4000 cm^{-1}.

4.3 Results and Discussion

4.3.1 SEM results for the produced multi wall CNTs

Scanning electron microscope was used to analyze the morphology and surface properties of the produced Mw-CNT. Figure 4.4.(a) shows the tube bundles agglomerated together forming ropes. Clear nanotubes with high purity as shown in figure4.4.(c) was produced using chemical vapor deposition. Figure 4.4.(b) shows catalyst particle (Ferrous) agglomerated after the decomposition of the ferrocene molecules. As the mixture of p-Xylene/Ferrocene was introduced to the reactor, and with the high temperature (850^oC), the iron atoms detached immediately form ferrocene molecules and agglomerate forming an active catalyst surface for the carbon source. The carbon atoms cracked from p-Xylene attacks the active Fe surface forming a start-up base where the CNT begins to be initiated [113, 114]. As shown from figure 4.4.(d) nanotubes are formed on the surface of the catalyst and starts to grow forming long tubes. The existence of Fe catalyst is very important for the process to complete. It enables the carbon active atoms to be formed in a tubular shape after it had been cracked from the liquid carbon source. Many, theories and methodologies was proposed to describe the growth of the nanotubes [115, 116].

Mo et al. (2001) proposed three steps that occur that results in the growth of the nanotube: (1) the carbon source (p-Xylene in our case) is adsorbed and dissociated on the active cites of the catalyst surface; (ii) in the second step the carbon atoms diffuses into the catalyst molecules due to the high activity; and (iii) finally carbon atoms precipitated at the surface of the metal particle forming nanotubes [117]. Figure

4.4.(d) shows typical representation of the steps mentioned, since tubes start to form on the surface of the catalyst after it was absorbed from vapor phase and precipitate forming the bundles. Figure 4.5 (a) and (c) shows clear tubes that obtained from CVD reactor. The produced bundles have high length up to few micrometers as shown in figure 4.5.(c). However using floating catalyst technique in the CVD reactor results in a random growth as observed in figure 4.5.(a). Uniform growth could be obtained if a substrate coated with catalyst used instead of haphazard catalyst agglomerated particles [113]. CVD technique produced Mw-CNT with diameters between 50 nm and 200 nm as shown from the labeled tubes in figure 4.5.(b). With this surface and morphology properties, the produced Mw-CNT are considered excellent support for lipase immobilization, which is the purpose of the synthesis.

4.3.2 Thermaogravimetric analysis (TGA) for the Mw-CNT and Mw-CNT-COOH

It is clear from figure 4.6 that the Mw-CNTs produced using CVD technique has high resistant to oxidation. From figure 4.6.(a), Green, degradation of the sample started at 500^oC which indicates high crystalinity for the CNT. The maximum oxidation rate occurs at 655^oC as indicated by the sharp peak in figure 4.6.(b), Blue. This is identical to what reported by previous studies for synthesis of CNT using CVD technique and similar conditions [96, 109, 118]. The high Mw-CNT resistance to thermal oxidation is due to structure of carbon atoms and its binding (hexagonal benzene ring), hence aromatic bonds are expected to be dominating [118]. The smooth thermogravimetric

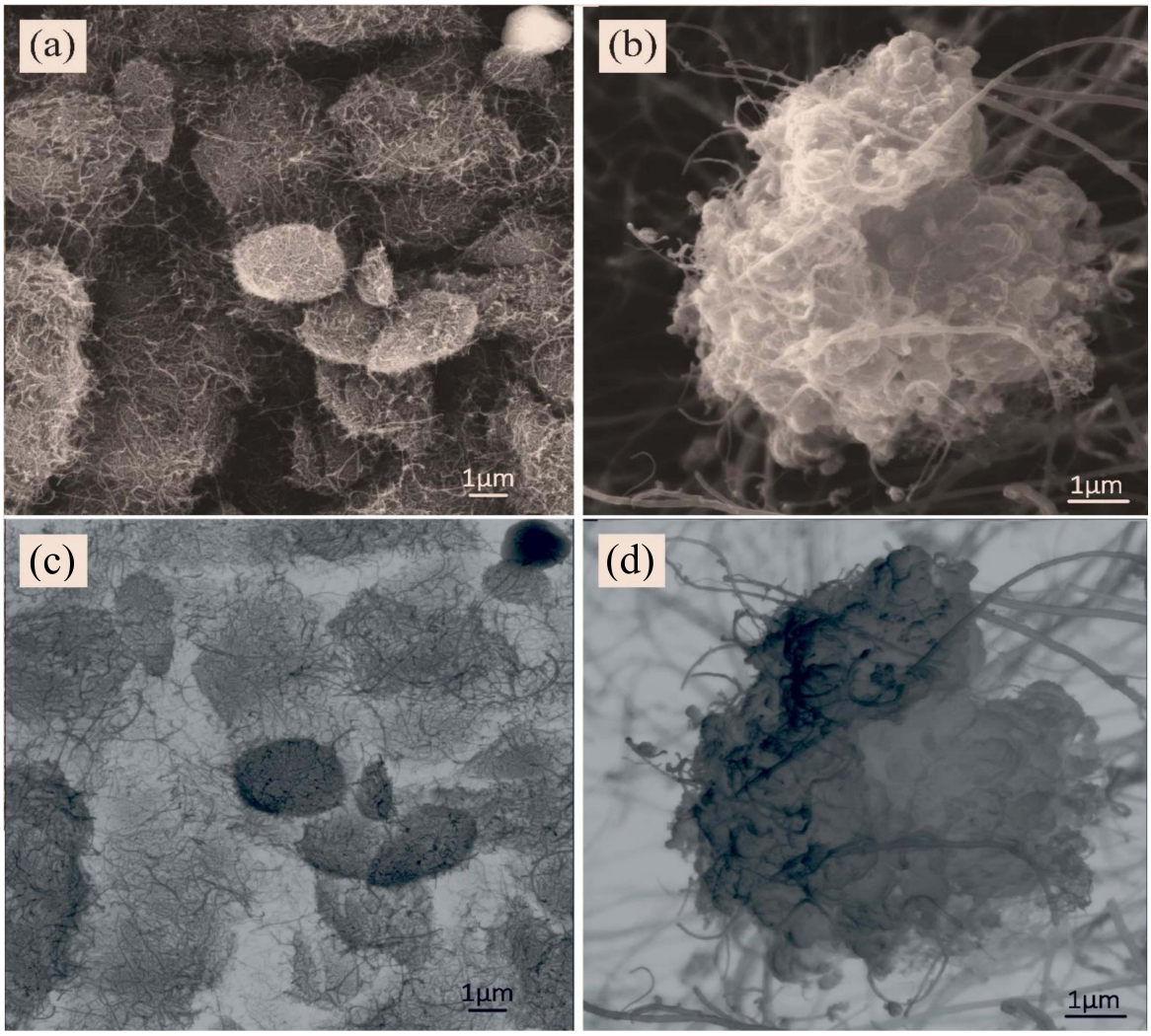

Figure 4.4: Scanning electron microscopy images for the produced Mw-CNTs. (a) CNT bundles agglomerated together, (b) catalyst particle after deposition, (c) and (d) same images inverted using image processing program. $HV : 15kV, WD : 11.30mm, Det : SE, MAG : 20kx$

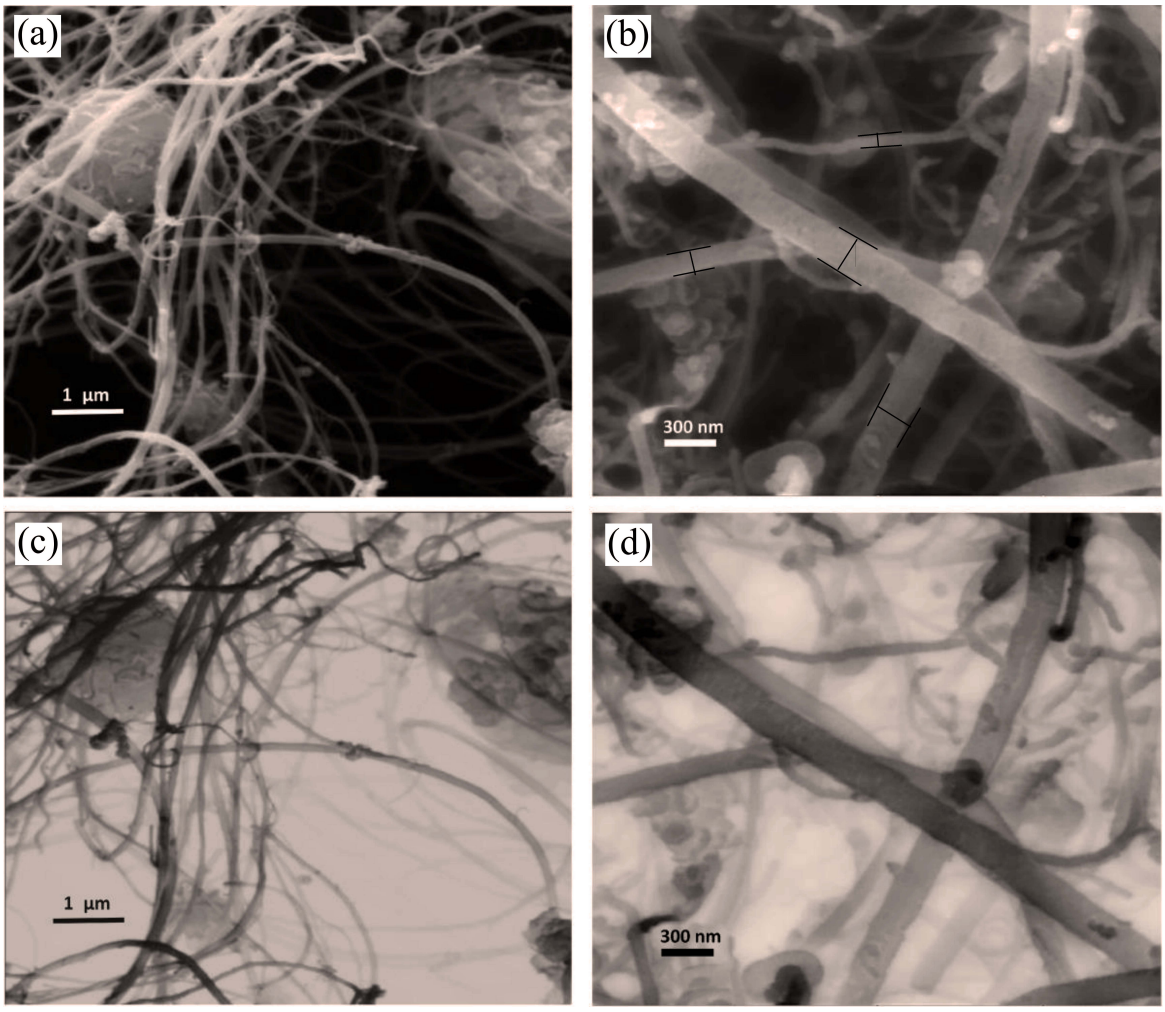

Figure 4.5: Scanning electron microscopy images for the produced Mw-CNTs. (a) and (b) is images as received from SEM device, (c) and (d) same images inverted using image processing program. $HV : 15kV, WD : 11.30mm, Det : SE + InBeam, MAG : 17.6kx$

degradation between 100^oC and 500^oC indicates the purity of the sample, thus no other forms of carbon or organic molecules appeared. However as indicated by figure 4.6.(a), Green, at the end of the thermal degradation ($700 - 800^oC$), the sample contains about $9.5\%wt$ of encapsulated iron particles (catalyst residue). Figure4.7 shows the thermogravimetric analysis for the Mw-CNT after treatment using nitric acid. The weight loss in the sample at 200^oC shown in figure 4.7.(a), Black, indicates to the degradation of carboxyl acidic groups. It is clear from the curve that at 400^oC 10% of the raw Mw-CNT was functionalized as carboxyl groups. Oxidation of CNT in the TGA analysis occurs and starts initially at tubes ends and open tips. Fractures and deflection of the nanotubes shell is possible to be functionalized and burned first. Functionalization using acids even breaks and make more deflections of the tubes shell to initiate carboxyl groups on the surface [119, 120]. These cracks and broken cites would easy the way for oxygen to be absorbed on that surface and burn the tubes. This phenomena explains the early degradation of the functionalized CNT sample at 450^oC as shown in figure 4.7.(a), Green, compared to 500^oC for the raw one figure 4.6.(a). Figure 4.7.(b), Blue, confirms the observed results, since the maximum oxidation of the functionalized Mw-CNTs occurs at 570^oC which is lower than what observed for raw CNT (655^oC) shown in figure 4.6.(b), Blue. It is important to notice that the acid treatment had removed the catalyst impurities from the samples as shown form figure 4.7.(b). At a temperature range between $700 - 800^oC$, impurities left in the pan after burning was $3\%wt$, while it was 10% as shown in figure 4.6.(a) before the acid treatment.

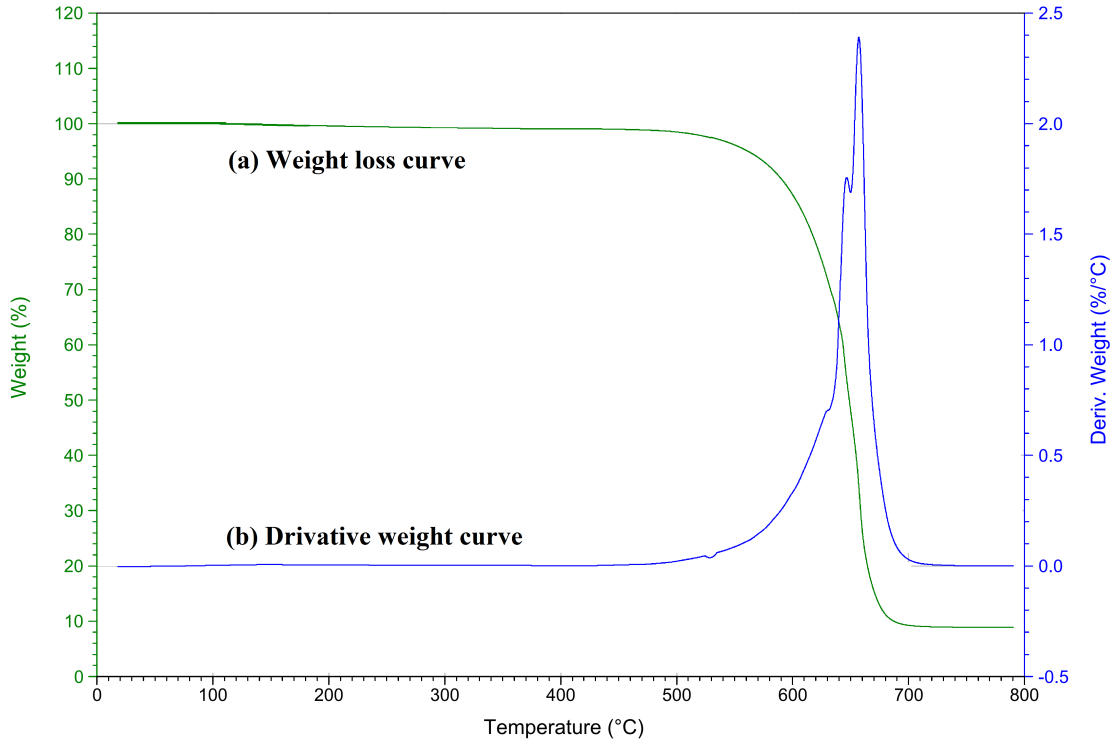

Figure 4.6: Thermogravimetric properties of the Mw-CNT material. Green curve (a) is the weight loss percentage, Blue curve (b) shows the derivative weight change. Ramp rate $1^oC/min$, Sample weight 7 mg, Air flow rate 100 ml/min

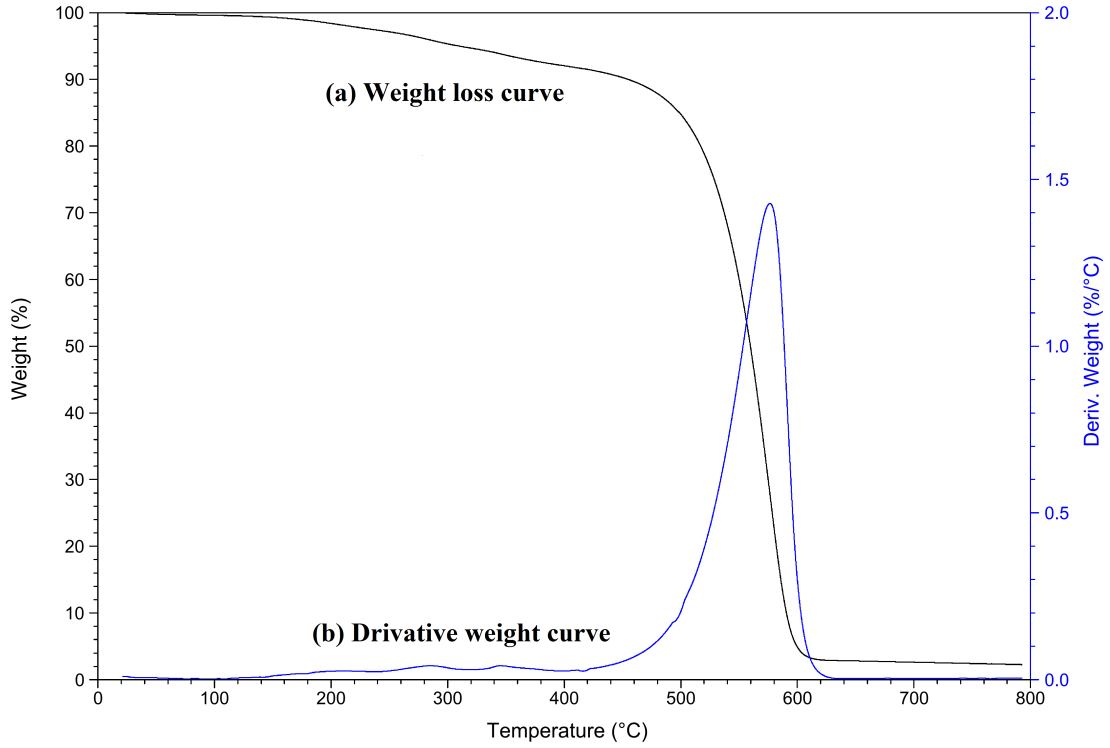

Figure 4.7: Thermogravimetric properties of acid treated Mw-CNT. Black curve (a) is the weight loss percentage, Blue curve (b) shows the derivative weight change. Ramp rate $1^oC/min$, Sample weight 7 mg, Air flow rate 100 ml/min

Fourier Transform Infrared Spectrometry (FTIR) results

FTIR was performed to investigate and confirm the oxidation of the produced CNT using acid treatment. Spectrum in figure 4.8 indicates the transmittance of infrared trough the sample and the consequent resonance on chemical bonds. A quick comparison of the FTIR spectrum (a) and (b) in figure 4.8 shows the difference in the sample before and after the treatment. Peaks raises at 1670 cm^1, 1180 cm^{-1} and 3360 cm^1 for treated Mw-CNTs using acid compared to the raw Mw-CNTs shows that acid oxidation has functionalized the surface of CNT with some groups. Acid strength and the reflux and treatment time is key factors that control the quantity of these functional

groups [110, 121]. The peak appears at 1670 cm^{-1} indicates the (C=O) stretching vibration of carbonyl groups associated with aromatic bindings [110, 121, 122]. While that at 1180 cm^{1} is due to C-O stretching and O-H bending vibrations [110, 121, 122]. As an overall conclusion, FTIR analysis provided strong evidence for the existence of carboxyl groups and acid defects in Mw-CNT walls.

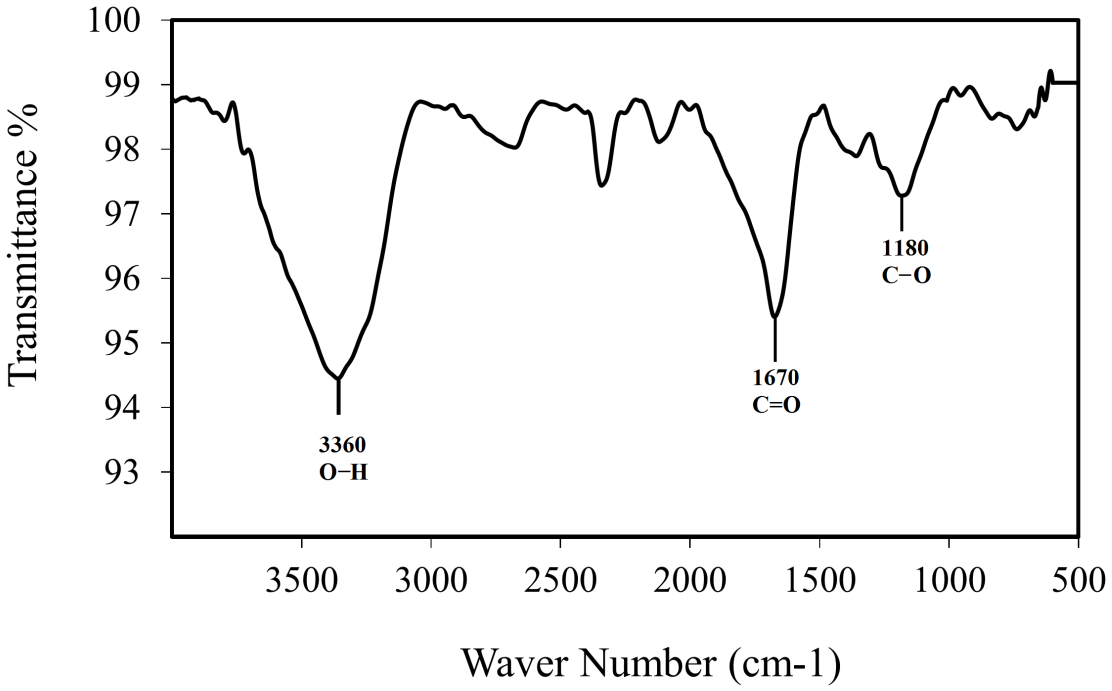

Figure 4.8: FTIR spectrum for acid treated Mw-CNT

4.4 Conclusion

In this chapter, multi-wall carbon nano-tubes (Mw-CNTs) was synthesized using chemical vapor deposition technique. The produced Mw-CNTs were found to have high aspect ratio when analyzed using scanning electron microscopy (SEM). Thermo-gravimetric analysis (TGA) results showed that the produced Mw-CNTs have purity up to 92%. Because ferrocene was used as a catalyst, SEM images showed some iron particles attached to the tubes bundles as impurities. The produced Mw-CNT was treated using Nitric acid oxidation. Fourier transform infra-red (FTIR) analysis confirmed the appearance of carboxyl groups bands in the samples. After analyzing the acid treated Mw-CNTs using TGA, results showed that up to 10% (wt) of Mw-CNT was functionalized into carboxyl groups.

CHAPTER 5

IMMOBILIZATION OF LIPASE ON MW-CNT FOR OILY WASTE WATER TREATMENT

Abstract

In this chapter, immobilization of lipase enzyme on multi-wall carbon nanotubes (Mw-CNTs) using covalent binding is investigated and studied. Using a chemical vapor deposition (CVD) technique, Mw-CNTs were produced and then treated by nitric acid and organic cross-linkers. The immobilization reaction takes place in an aqueous phase using buffer solutions with adjusted pH. Fourier transform infrared (FTIR) and Thermogravimetric analysis (TGA) show suc¬cessful attachment and high enzyme loading up to 19.5% (wt). Immobilization of lipase on Mw-CNTs enhanced the catalytic activity of the enzyme when tested in oil/water emulsions. The titrimetric analysis of hydrolyzed samples using MwCNT-Lipase (after 1 hr reaction time at 37^oC) shows an

increase in the enzyme activity up to five times compared to the free lipase. Lipase activity and enzyme loading depends on the oxidized Mw-CNT surfaces, cross-linkers type and concentrations, and enzyme amount. The biomaterial shows high thermal and operational stability and activity when tested in oil/water emulsions prepared in the lab, and can resist the severe conditions in industrial applications.

Keyword: FOG, Lipase Immobilization, Carbon nanotubes, Wastewater Treatment

5.1 Introduction

Fats, Oils, and grease, refers as "FOG", are one of the main water pollutants coming from food industries, restaurants, slaughterhouses, leather processing units, and dairy waste water [2–4, 123]. Many problems that face waste water plants when handling the oily or fatty polluted water. Mainly, waste water contains another pollutants including salts and minerals, not only it is considered as hazardous materials, but also it works as emulsifiers and helps the oil to be blended into the water forming different complex oil in water emulsions [124]. Moreover, because of the heavy viscous property of FOGs, it clogs the networks in waste water treatment plants causing additional maintenance concerns. Furthermore, the floatation of oils on the water/air interface causes real issues in activated sludge processes and biological treatment, since it limits the oxygen transferred into the water to activate the biomass [6, 125]. Concurrently, micro-organisms and bacteria used in these processes adsorbs oils and greases which make it float to the surface reducing the biological treatment efficiency.

Additionally, fatty acids resulted from FOG esterification and hydrolysis inhabits the bacteria and micro-organisms activity [7]. Beside these severe treatment technology concerns, FOG causes serious health and environmental problems by blocking sewer pipes and reducing its diameter, it causes flooding and attract pathogens and vermin [8].

There are many methods and techniques that are used in the waste water treatment [126–130]. The preference of one to the other depends on many factors including the source of the oily water, the contents of the pollutants, and the usage of water after treatment. The removal of oil and the treatment efficiency is determined by the concentration and physical properties of the oils existing and its drop size [124]. From the many methods that are used to deal with such waste water including chemical, physical, and biological methods [126], enzymatic hydrolysis for the oily waste water comes in the forefront [127]. Enzymes like lipases and esterases have been used widely in last decades in the biochemical processing of FOGs, hence, it degrades and transform the complex fatty triglycerides into simpler chains of free fatty acids [131]. As mentioned, lipase has very important role in catalyzing many significant reactions. Including hydrolysis of triglycerides it plays major role in waste water treatment applications, esterification, transesterification, synthesis of peptides, region selective acylation of menthols and glycols, and production of many important chemicals [56]. These vital reactions put lipases in the forefront of the promising materials that could be used in biotechnology and industrial applications such as pharmaceutical synthesis, detergents, cosmetics, food industries, organic chemical processing, nutrition and

agro-chemical industries bio-surfactants, and recently oily waste water treatment [34]. Nonetheless, all these applications, which include severe industrial conditions specially waste water treatment, is often restricted by the deficiency in long time operational stability, durability and the difficulty to recover and recycle the enzymes [56]. These disadvantages can be overtaken by using enzyme immobilization [132].

Mainly, attaching enzymes to a certain support has many advantages. Support matrices have chemical characteristics and mechanical properties such as strength, temperature handling, surface area, thermal and electrical conductivity and the most important is the ability to be fabricated in different reactor processes including packing, fluidization, immersing and recycling. On the other hand, enzymes have another reactive and biochemical properties that controls the catalytic process and the type of kinetics and it determines the final products. Combining both properties provide many merits to the whole process. Hence, the operational stability is the most important key factor for any catalyst used in the industry, the support increases the durability and the mass transfer effect maintain the high efficiency for the immobilized enzyme. However, the immobilization method controls the yield and the reaction performance since it affects the chemical characteristics of the enzyme.

Many organic and inorganic supports have been used for lipase immobilization [133]. Recently, the use of nano-materials as a supports for the enzyme immobilization has received significant attention. It is well known that many nano-materials has very large surface area which can provide large enzymes loading and also it has many physical, mechanical and electrical properties made it in the forefront of the global

research interest [14, 15]. Carbon nanotube which is a nano-scale one dimensional tubular material has a graphitic structure is considered one of the most famous nano-materials that can be used as a support for enzymes immobilization. Considering its high aspect ratio and significant mechanical properties it is expected that it would be a good support matrix for enzyme loading [10]. In this study, considerable effort was focused on the study of the covalent immobilization of lipase on the surface of the multi-wall CNTs. The immobilized lipase was applied and used in oily waste water degradation and treatment.

5.2 Materials and Methods

5.2.1 Materials

Candida Rugosa Lipase, N-ethyl-N-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES), N-hydroxysuccinimide (NHS), and Gum Arabic were purchased from Sigma Aldrich Co. US. Purified high quality olive oil was supplied locally from Hail Agricultural Development Co. KSA. Absolute Ethanol, Hydrochloric Acid, Sodium Hydroxide, phosphate buffer that was used were supplied from local companies with laboratory grads. High purity functionalized CNT (MwCNT-COOH) was supplied from Nanostructured and Amorphous Materials Inc. US. As described in Chapter 3.5, high quality multi-wall carbon nanotubes was produced locally using CVD technique described in section 4.2.1. The produced Mw-CNT was then treated and prepared with acid oxidation for the purpose of using it

as support matrix for the enzyme. After treatment and characterization using SEM, TGA, and FTIR, the produced Mw-CNT was found to be with an excellent quality compared to the commercial CNT purchased. Confidently, it can be used as a support for the lipase immobilization.

5.2.2 Immobilization of lipase on the functionalized CNTs

40 ml of 50 mM $2-(N-morpholino)ethanesulfonicacid$ MES) with pH of 6 was added to 40 ml of 400 mM $N-Hydroxysuccinimide(NHS)$ solution in MES. The mixture was added to 80 mg functionalized carbon nanotubes (FCNT). The mixture was sonicated for 30 min (Amplitude 50% and 0.8 $Cycles$), followed by addition of 40 ml of 20 mM $1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC)$ and the whole mixture was stirred for 30 min at 200 rpm. Using filter paper and vacuum filter the reaction mixture was filtered and washed using MES buffer. The sample after filtration was about 0.15 g. Lipase enzyme was added after this step. The pretreated sample was added to 20 ml of 10 mg/ml lipase and sonicated for 3 min. The mixture was shaken at 200 rpm overnight and then filtered using the vacuum drier and washed three times with deionized water.

5.2.3 Immobilized lipase characterization and analysis

Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopy (SEM) was used to study the morphology of the samples. SEM images for the tube bundles before and after the immobilization was taken.

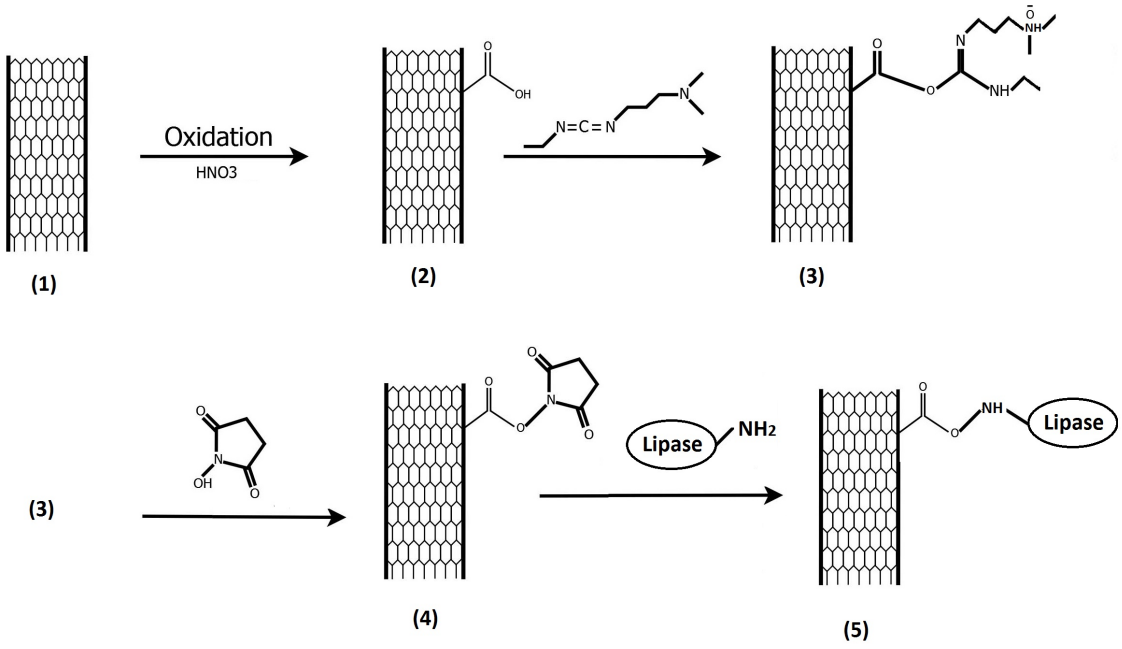

Figure 5.1: Covalent attachment of lipase on Mw-CNT surface: (1 to 2) Acid oxidation using HNO3 to make carboxyl groups on the CNT surface, (2 to 3) Addition of EDC cross-linker to activate the carboxyl group, (3 to 4) Attaching an amide good leaving group (NHS) making the semi-stable intermediate (CNT-NHS), (4 to 5) Replacing NHS group with the lipase amide functional group making the stable covalent bond

Different magnifications and electron beam energies were used during the analysis to investigate the existence and attachment of the enzyme to the nanotubes walls.

Energy dispersive X-ray spectroscopy (EDX) analysis was the key factor for the elemental composition for the samples. After analyzing the surface using the (SEM-EDX), samples were characterized and the elemental composition before and after the immobilization was measured. Three kind of samples were investigated: (1) pure functionalized (oxidized) Mw-CNT before the immobilization; (2) free lipase enzyme as received from the supplier company; and (3) the immobilized lipase on Mw-CNTs after the functionalization. Comparing the elemental content of the three samples can give us very clear image about the attachment.

Thermogravimetric Analysis and characterization

Thermogravimetric analysis (TGA) was performed for the samples to investigate the evaporation and burning of the bonds that indicate the attachment efficiency and stability. Studying the degradation of Mw-CNT before and after the immobilization and in comparison to the free lipase will give a clue about the attachment success. This test was done using a TA Q500 thermogravimetric Instrument as described in section 4.2.3. Sample was prepared and 7 mg was taken into a platinum pan. Heating rate was about $1^oC/min$ and the maximum temperature was adjusted to be 900^oC. The test was performed in air flow of $100ml/min$ through the burning period.

Fourier transform infrared spectrum Analysis

As an important analytical tool for molecules alteration and chemical binding modifications Fourier Transform Infrared spectroscopy (FTIR) was used to analyze the samples before and after the immobilization reaction. Since it is useful as a direct analyzing method for the modified surfaces nature, it should be considered as a basic tool when coming to enzyme immobilization. Though its importance, however it has some experimental limitations [110]. When coming to carbon materials characterization, it could only be used for material with high oxidized surfaces since the bands absorption intensity will be very low. As descriped in section4.2.3, a factor that has hindered the advancement of FTIR as a tool for Mw-CNT analysis is the poor infrared transmittance of Mw-CNTs [111]. Potassium bromide (KBr) was used to dilute the samples and to allow more IR to pass throw as recommended by [112]. After grinding of Mw-CNT, lipase, and MwCNT-lipase with potassium bromide, plates with a suitable size for the device were made for each sample using manual press. Typically, 16 scans with a wave length range from 500 cm^{-1} up to 4000 cm^{-1} were used to cover the full range of bonds resonance.

5.2.4 Immobilized lipase activity

The immobilized enzyme activity was investigated based on the hydrolysis of the emulsified olive oil as described in section 3.3.3. Olive oil/water was prepared to simulate the real oily waste water. Liberated fatty acids was then titrated with NaOH 0.05N to calculate the initial rate of reactions [61]. The volume of NaOH used represents the

quantity of liberated fatty acids form the hydrolysis process. Olive oil/gum Arabic substrate was used in the hydrolysis reaction [61]. To prepare the substrate, 10 g each olive oil and gum Arabic was added and mixed in a 400 ml beaker and the volume was brought to 200 ml using 50 mM sodium phosphate buffer, pH 8.0, and homogenized for 5 min using a domestic blender without causing excessive foaming. The MwCNTs-Lipase was added to the substrate and sonicated for $3min$ to ensure that it has been dispersed homogeneously. The lipase activity is measured by the lipase units LU, which is defined as the quantity of lipase that can degrade 1 μmol of olive oil per min at the standard reaction conditions recommended in the literature (temperature of 37^oC, Olive oil/gum Arabic substrate, agitation speed 200 rpm, and Sodium Phosphate buffer with pH 8.0) [61]. Equations (3.10 and 3.11) shown in section 3.3.3 represents the calculations of lipase units and specific activity.

5.3 Results and discussion

5.3.1 Scanning electron microscopy (SEM) Analysis

Figure 5.2 shows SEM images for lipase crystals with different magnifications. From image (b) in figure 5.2, it is clear that lipase has a shiny crystals structure. From shown in figure 5.3.(a), shiny parts on surface of the tubes bundles indicates the existence of another brighter material on the surface. This images gives us an indication of lipase molecules attached to the surface. Using higher magnification, Image in figure 5.3.(b) shows clear enzyme molecules attached to the Mw-CNT surface. This bright

structures was noticed along the tubes and in the fractured parts of the CNTs.

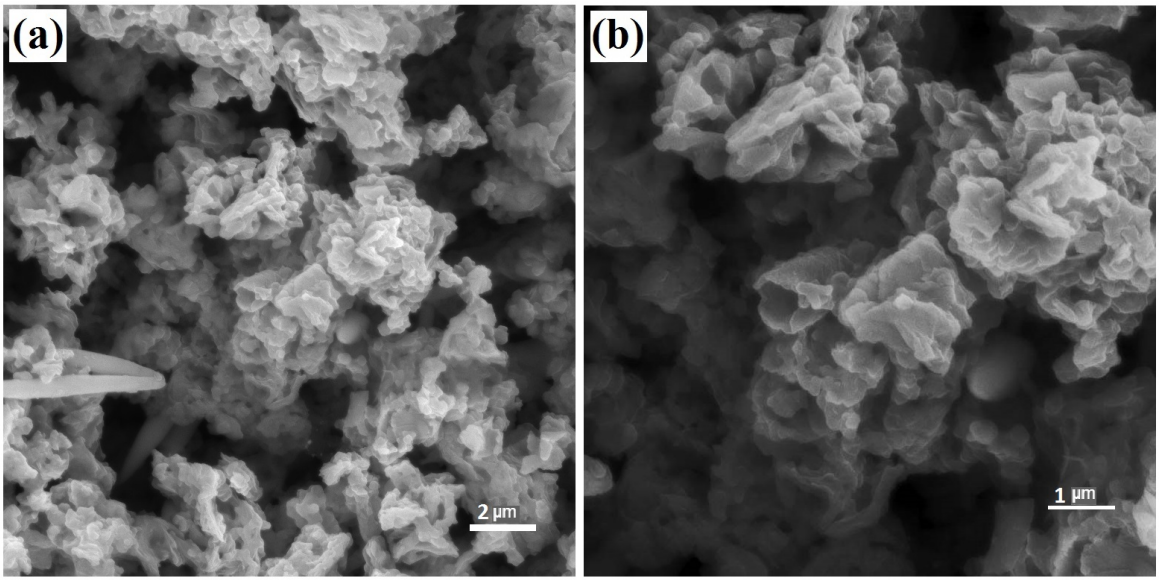

Figure 5.2: Scanning electron microscopy image for the free lipase, HV : $30.0kV, MAG : 80kx, Det : SE$

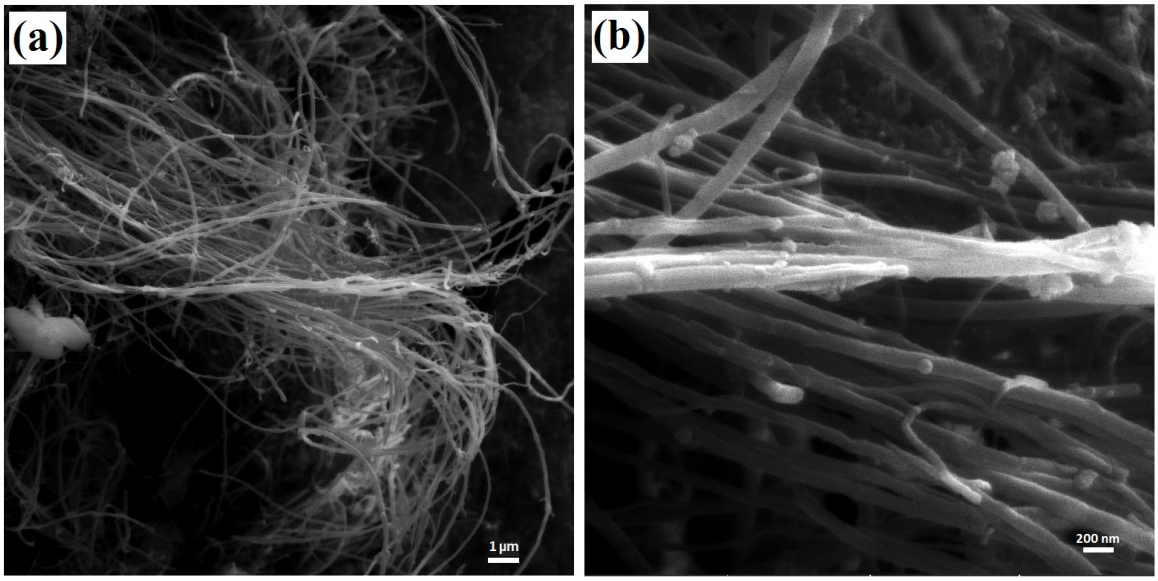

Figure 5.3: Scanning electron microscopy SEM image for the Immobilized lipase on the MW-CNT surface, $HV : 30.0kV, MAG : 80kx, Det : SE$

5.3.2 Thermaogravimetric analysis (TGA)

Thermal degradation results for free lipase, Mw-CNT, and MwCNT-Lipase were collected and analyzed as shown in figure5.4. figure 5.4.(a), shows smooth degradation under thermal conditions when temperature reached 500^oC, where the sample starts to burn and only catalyst particles (ferrocene) and ashes remained in the pan which was $7.3\%wt$. Free lipase sample figure 5.4.(c) shows small degradation ($4\%wt$) at the beginning of the burning below 100^oC. This is due to moisture evaporation from the sample as it starts to lose some weight because of drying. As the temperature increases the sample start to degrade again hence after 200^oC sharp decrease of enzymes weight happened due to chemical bonds disintegration. After 350^oC burning rate was slowing down notably until it remained constant at $50\%wt$ to the end of the process. Comparing immobilized lipase sample with previous results it is clear that at the beginning of heating $10\%wt$ of sample weight was lost due to moisture evaporation.

When temperature raised to a range between 225^oC and 550^oC, rapid decrease occur, leaving only 70%wt of the sample. This last weight loss ensures the enzyme attached to the CNT when was compared to Mw-CNT curve (a) and pure lipase (b) at the same range. Since no change to Mw-CNT sample happened at the same range and for free lipase it shows similar behaviour and trend as for MwCNT-Lipase sample. From last observation of figure 5.4.(c), at the range between 225^oC and 540^oC we can ensure that 19.5% wt of lipase was attached to the Mw-CNTs if we compare it with figure 5.4.(a). According to the literature, Pavlidis et al. (2010) studied covalent attachment of lipase on Mw-CNT using organic cross linkers. Our findings

are very close to what they got since they reported 25%wt of enzyme attachment to the CNT [10].

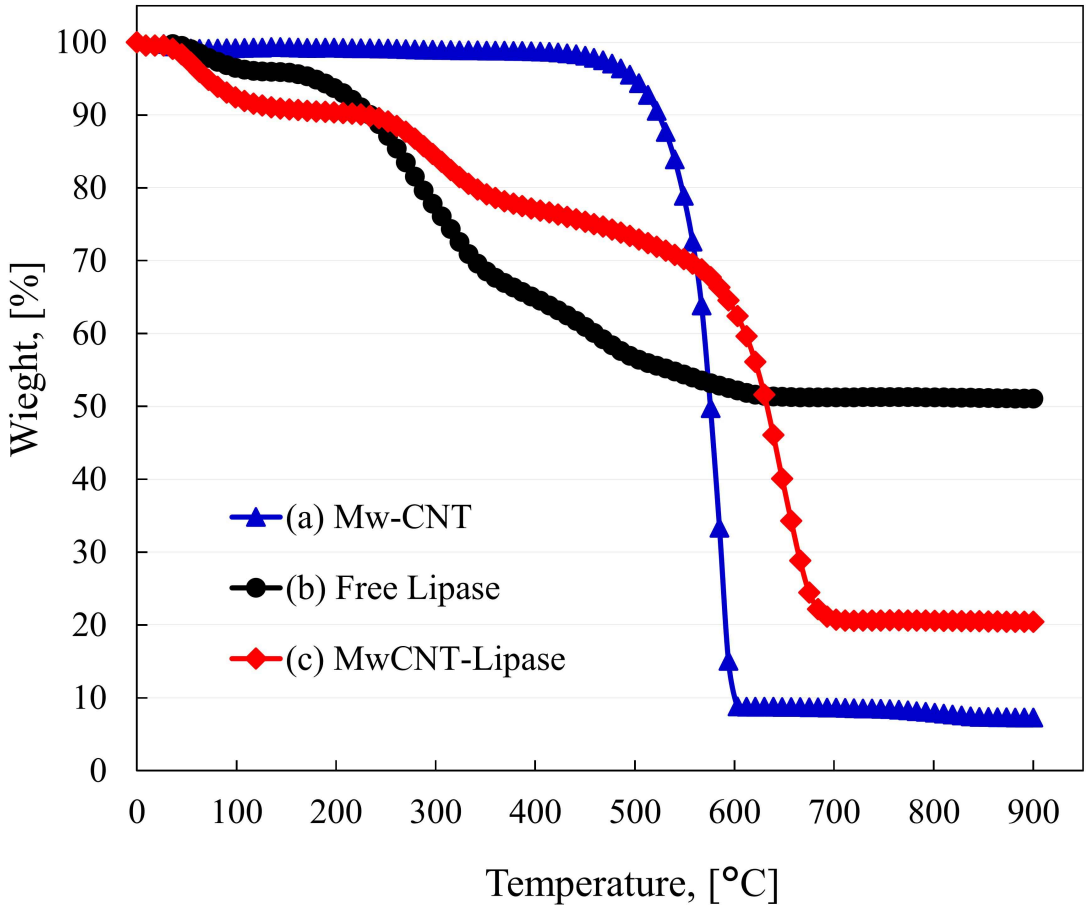

Figure 5.4: Thermogravimetric analysis (TGA) for Mw-CNT (a), Free Lipase (c), and Immobilized lipase (b). Samples weight= 7 mg, Platinum pan, Heating rate = $1^oC/min$, Maximum temperature, (T_{max})= 900^oC, Air flow rate = $100ml/min$.

5.3.3 Energy dispersive X-ray (EDX) results

Energy Dispersive X-ray spectroscopy (EDX) analysis was conducted on the samples to check the elemental composition before and after the immobilization. Table 5.1 shows that some elements had appeared after the immobilization, this confirms the

existence of the enzyme in the immobilized sample. Pure CNTs contains 90% carbon as expected while the free lipase has about 64.6%. Therefore, results for immobilized lipase sample shown 72.64% of carbon content. Oxygen content has increased from 8.2% in pure CNTs to 24.79% in the immobilized lipase sample which indicates clearly the appearance of new materials to the sample. For pure lipase oxygen content was found to be 31.42%, this is explains the increase of oxygen percentage in MwCNT-Lipase sample. Other elements like Magnesium (Mg), Phosphorous (P) and Calcium (Ca) appeared in the MwCNT-Lipase samples confirming the attachment of lipase to the nanotubes as shown in table 5.1.

Table 5.1: EDX results and the atomic percentage of three samples: Functionalized carbon nanotubes (Mw-CNT), pure lipase (Lipase), and Immobilized lipase (MwCNT-Lipase)

Element	Mw-CNT	Lipase	MwCNT-Lipase
C K	89.99	64.59	72.64
O K	8.20	31.42	24.79
Mg K	-	0.54	0.36
P K	0.35	0.98	1.27
Cl K	0.14	-	-
K K	0.14	1.45	0.07
Ca K	-	0.38	0.80

5.3.4 Fourier Transform Infrared analysis

Fourier Transform Infrared spectroscopy (FTIR) was performed to investigate the covalent binding between lipase and oxidized CNTs. Spectrum shown in figure 4.8 specifies the transmittance of infrared trough the samples before and after the immobilization. From figure 5.5 we can clearly notice that for oxidized Mw-CNTs spectrum

(a), band at 1680 cm^{-1} indicates the carbonyl (C=O) stretching vibration associated with aromatic bindings [121, 134, 135]. While that at 1180 cm^1 is due to C-O stretching and O-H bending vibrations [110, 121, 122]. The existence of hydroxyl broad peak at 3350 cm^{-1} confirms the oxidation of Mw-CNT. For Free lipase spectrum figure 5.5 (c), double peaks appeared between 1600 cm^{-1} *and* 1700 cm^{-1} exactly at 1645 cm^{-1} *and* 1750 cm^{-1} indicates primary amide region. Band appeared at 3450 cm^{-1} is due to amide stretching (N-H) which confirms the amide existence. The sharp peak in MwCNT-Lipase spectrum appeared at 1607 cm^{-1} indicates the carbonyl binding to the amide [10, 121]. It differs from the previous one noticed for pure Mw-CNT and lipase since it was shifted from 1680 cm^{-1} (on MW-CNT sample) to 1607 cm^{-1}. This shift is due to the chemical bond between the amide and the carbonyl, since the acetamide binding decreases the carbonyl resonance to IR that have higher wave length. The sample also shows clearly the symmetric and asymmetric band vibrations of $(-CH_2-)$ groups at 2870 and 2930 cm^{-1}, respectively. These groups was not clear in oxidized MwCNT spectra, verifying the successful attachment of organic groups to the carbon nanotubes [10, 121]. The appearance of sharp peak nearly at 3430 cm^{-2} confirms amide existence in the sample. The small shoulder noticed at 3200 cm^{-1} indicates the remaining traces of hydroxyl group. FTIR analysis confirmed the immobilization of lipase to Mw-CNT indicating clear covalent binding between the enzyme and the nanotubes surface.

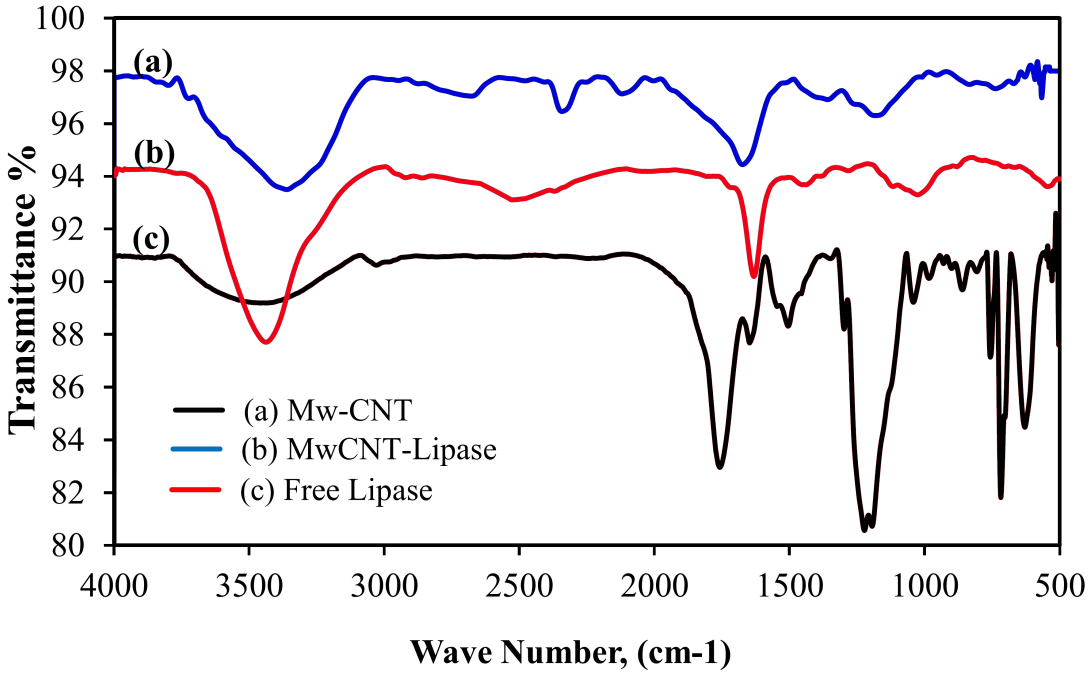

Figure 5.5: Fourier transform infrared spectrum for oxidized carbon nanotubes, free lipase, and Immobilized lipase on MwCNTs

5.3.5 Immobilized lipase activity

Immobilized lipase activity was measured in olive oil emulsions. Figure 5.6 shows lipase units LU which represent the initial rate of reaction for immobilized lipase. Immobilized enzyme exhibits higher activity reaches up to 35 LU after only 20 min, but it decreases dramatically until it reach 8 LU after one hour reaction time. Krakowiak et al. (2003) studied the immobilization of lipase on chitosan polyphosphate porous beds and they found that the activity of the enzyme decreases when they used it for olive oil hydrolysis. They reported 3.7 U/ml for Chitosan-Lipase composite which is even incomparable to what is shown in figure 5.6 since the MwCNT-Lipase activity reached up to 345 U/ml [74].

Figure 5.7 shows fatty acids liberated by free lipase compared to immobilized li-

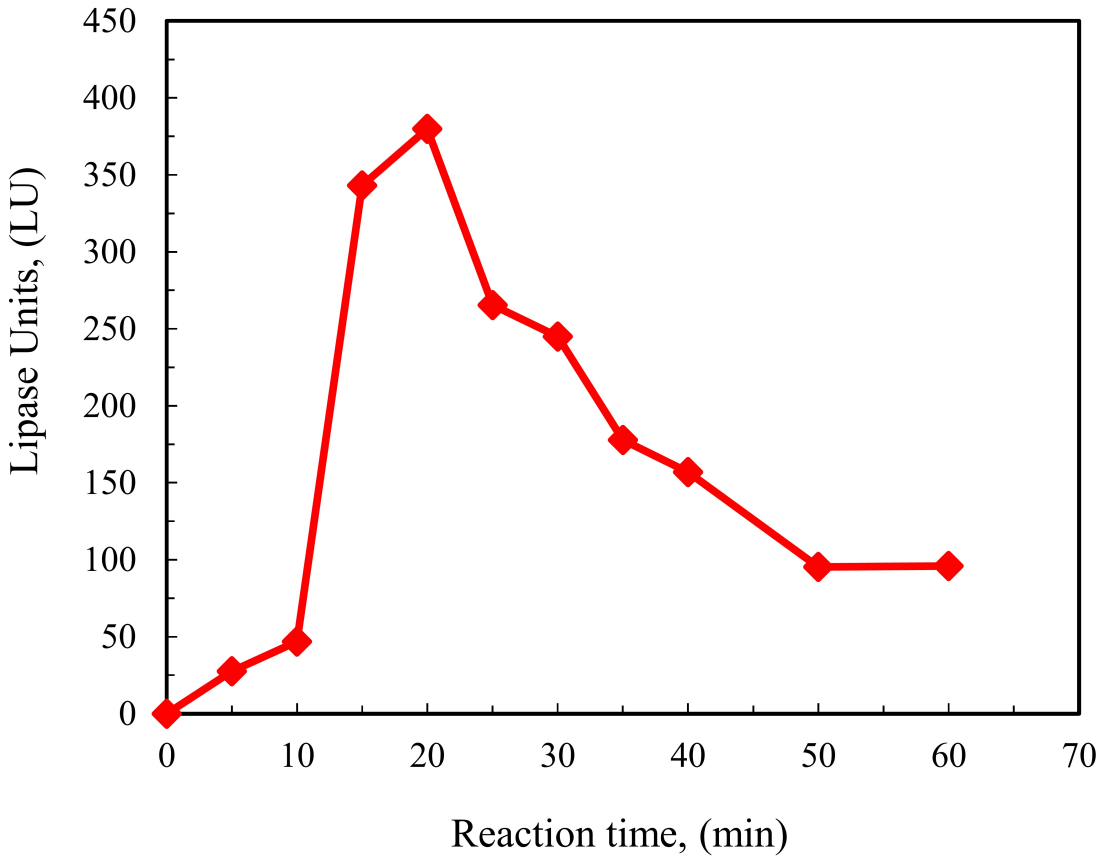

Figure 5.6: The activity of immobilized lipase on the CNTs measured in LU, Olive oil/gum Arabic emulsion substrate, Temperature=37.0^oC, agitation speed 200 rpm, and Sodium Phosphate buffer with pH 8.0)

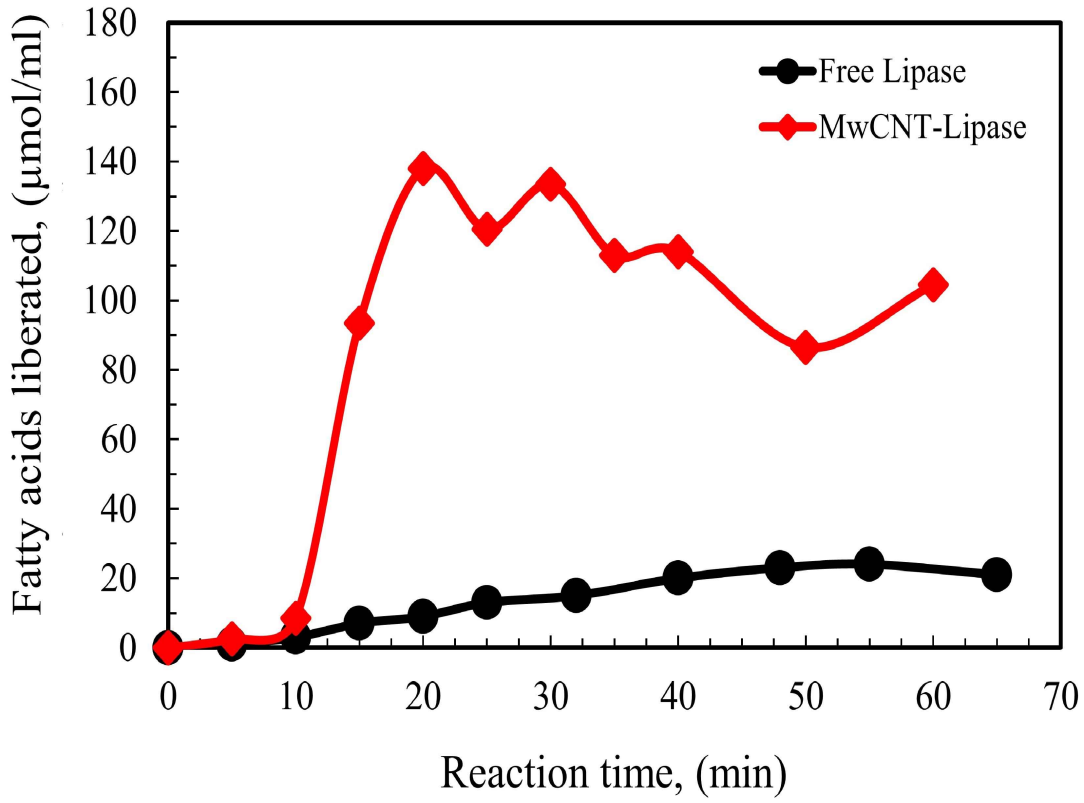

Figure 5.7: Effect of reaction time on the amount of fatty acids liberated by free lipase (Black) and MWNT–lipase (Red), enzyme Loading = 1 mg/ml, olive oil/gum Arabic emulsion substrate, pH = 8, reaction temperature = $37.0^{o}C$, mixing speed =200 rpm

pase (MwCNT-Lipase) at similar reaction conditions. Results shows that immobilized lipase has an average of nearly 100 $\mu mol/ml$ along the first hour of the reaction, while free lipase shows lower activity reaches up to 20 $\mu mol/ml$. As shown in figure 5.7 after 20 min reaction time the amount of fatty acids liberated reached up to 140 $\mu mol/ml$ or 140 $\mu mol/mg\ lipase$ considering $1mg/ml$ as an enzyme concentration.

The significant increase in immobilized lipase activity in contrast to free lipase may refer to Mw-CNT hydrophobicity nature. Recently, many authors reported that increasing the hydrophobicity support material used for immobilization of an enzyme may increase its activity by repairing and reforming the enzyme functional structure [10, 136]. Menaa et al. (2008) studied the encapsulation of lipase into modified silica glasses and they found significant increase in the enzymes activity due to attachment to such hydrophobic matrix. They reported an increase in enzymes activity when changing glass hydrophobicity. This change in hydrophobicity could increase the properly-folded lipase fraction and regulate the substrate partitioning into the support material [136].

Mw-CNTs creates a hydrophobic environment with oils esters affecting the substrates and products diffusion and distribution along the hydrolysis process. The hydrophobic micro- environment of the MwCNT-Lipase created after the attachment increases the openness to oil substrates, which is naturally hydrophobic, and thus enhances the esterification and lipase activity [10]. According to Sibel Fadıloglu and Zerrin Soylemez (1998), they studied the hydrolysis of olive oil using lipase immobilized on Celite and reported 180 $\mu mol\ fatty\ acids/m\ lipase$ in similar reaction

conditions after 20min reaction time. However, considering the substrate concentration that they used our immobilized enzyme shown better results. Since they used 12.5%(v/v) comapred to only 2.72%(v/v) that we used.

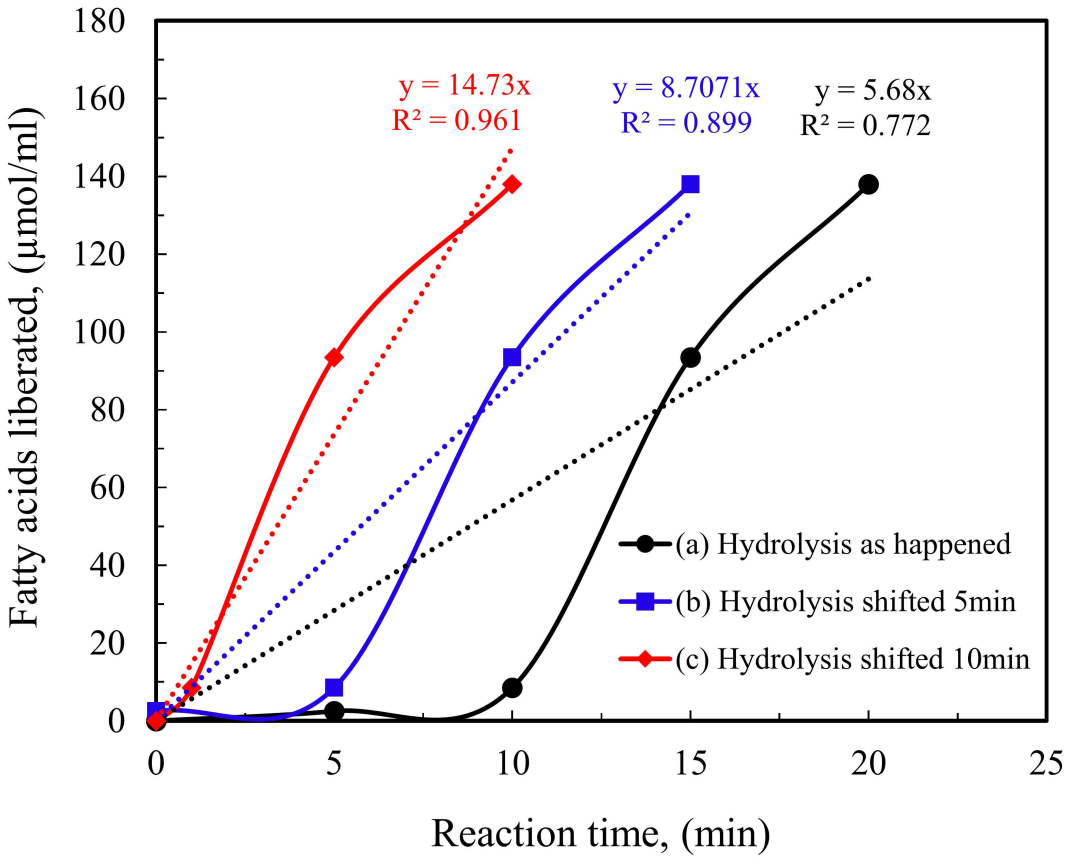

Figure 5.8: Initial velocity calculations and linear fitting for immobilized lipase activity (MwCNT-Lipase). Reaction start point assumed to be: (1) At the beginning of agitation (Black); (2) After 5 min; and (3) After 10 min of mixing. Conditions: Enzyme Loading = 1 mg/ml, Olive oil/gum Arabic emulsion substrate, pH = 8, Reaction temperature = 37.0^oC, Mixing speed =200 rpm.

To calculate the specific activity (sp_{act}) of the enzyme under the conditions stated, as described by equation 3.10 in chapter 2.4, initial velocity (v_0) for the reaction should be obtained. From figure 5.7 by looking to the immobilized lipase curve (Red), it is clear that the reaction had started slowly and after 10 min sudden jump occurred.

This is due to the agglomeration of the Mw-CNT bundles that envelopes the enzymes and retard it from the substrates. After a period of time while mixing it was detached and the enzyme was exposed and started to interact with the substrate. Therefore, for the calculations of the initial velocity (v_0) this elapsed time should be ignored. This is because initial velocity calculations only considers the linear portion that appears at the beginning of the reaction [61]. Figure 5.8 shows the initial traces of fatty acids produced form olive oil hydrolysis using the immobilized lipase. figure 5.8. (a) shows cut of results from figure 5.7 in the range below 20 min reaction time. It is very clear that reaction rate stays very low (approximately zero) in the first ten minutes. This due to initial mixing time delay and in-homogenous nature of reaction mixture at the beginning. Suitable assumption for such case is to assume a delay time for the reaction and thus should be considered when calculating the initial velocity, v_0. Calculating of initial velocity without considering such important observation would result in wrong and fake enzyme activity. From figure 5.8.(a), estimation of v_0, (which represented by the linear fitting slope), shown that the initial velocity was 5.68 with poor R^2 (0.772). This estimation resulted in specific activity (sp_{act}) of 31.27 $\mu mol/mg \times min$ as calculated from equation 3.10. By shifting the reaction time 5 min forward as shown by figure 5.8.(b), the initial velocity increases since the data fits the linear approximation more accurate. Therefore, considering 10 min elapsed time as reaction pre-incubation period, initial velocity estimation increases up to 14.72 as shown in figure 5.8.(c). Considering the last assumption (10 min incubation time), the specific activity estimated and was found to be 81.1 $\mu mol/mg \times min$.

5.3.6 Effect of reaction temperature on immobilized lipase

The immobilized lipase was investigated and tested in a temperature range between 20^oC to 40^oC and enzyme activity was measured in comparison with the free lipase. Figure 5.9 shows the effect of temperature on MwCNT-Lipase activity compared to free lipase. Results shows that immobilization of lipase on to carbon nanotubes had improved the enzyme activity slightly compared to free lipase. Previous study by Karkowiak et al. (2003), mentioned a decrease in lipase activity when immobilized into supports [74]. Compared to results shown in figure 5.9, the activity of the enzyme remained nearly constant with slight decrease in low temperature range below 25^oC. This decrease may refer to the nature of oily substrates and its behavior at low temperatures. previous studies reported that the lipase activity depends highly on the solubility and viscosity of the substrate compared to water [137]. It is well-known that at low temperatures, oil viscosity and density increases severely forming agglomerated particles which is difficult to be absorbed to the enzyme active cites.

5.3.7 Synthetic waste water sample and enzyme lipolysis

Synthetic waste water was prepared by mixing olive oil with the tap water and gum Arabic to make emulsified phase. figure 5.10.(a) shows the sample after treatment with the free lipase. Clearly we can see the oil droplets on the surface of the water after it has been degraded by the enzyme. Figure 5.10.(b) shows the same water sample treated with the MwCNT-Lipase. Due to the hydrophobic behavior of the CNTs, the liberated oil is agglomerated with the MwCNT-Lipase nano-composite and

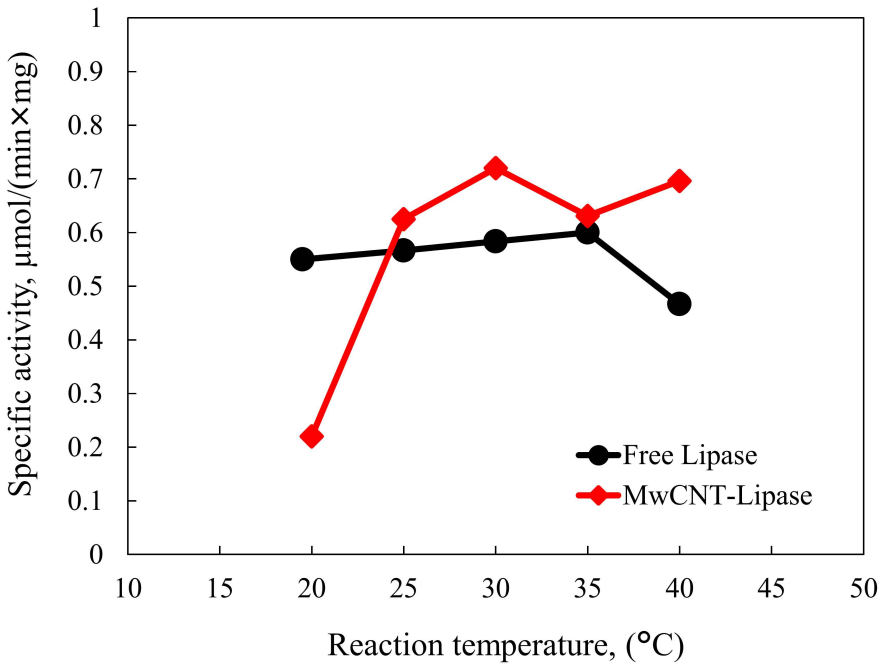

Figure 5.9: Effect of reaction temperature on the specific activity of the free lipase and MWNT–lipase, Enzyme Loading = 1 mg/ml, Olive oil/gum Arabic emulsion substrate, pH = 8, reaction time = 30 min, mixing speed =200 rpm

suspended on the surface of the water. This could be an advantage for the industrial applications that requires high enzyme loading for waste water treatment and gives us the opportunity to recycle and reuse the enzyme many times.

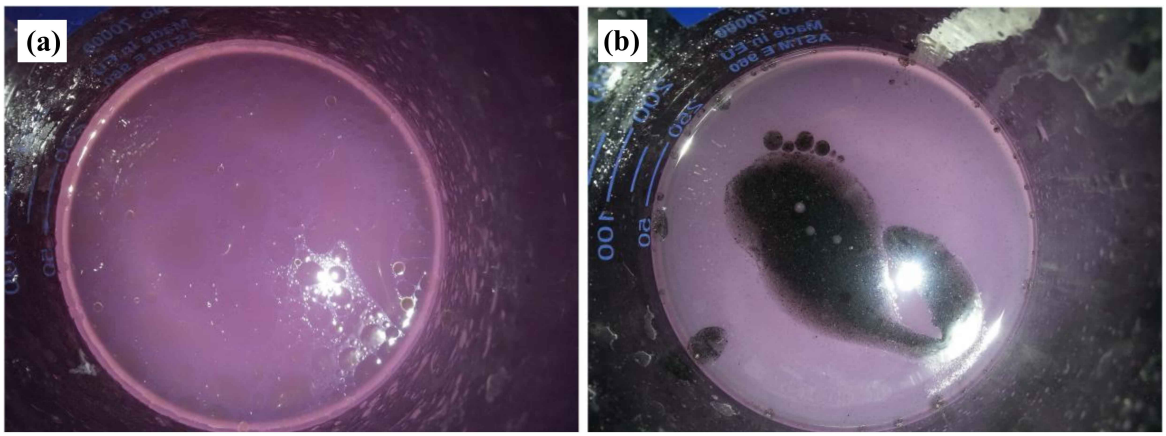

Figure 5.10: Synthetic waste water treatment samples. Image (a): Sample treated using free lipase, Image (b): Sample treated using MwCNT-Lipase. Conditions for each: enzyme loading [E_t]= 1 mg/ml, Temperature [T] = 37.0^oC, agitation speed [ω] = 200 rpm, reaction time [t] = 30min

5.4 Conclusion

We have immobilized the lipase enzyme from *CandidaRugosa* on the surface of Mw-CNT that is produced locally. Covalent binding using the cross linkers (NHS and EDC) was formed. The carbon nanotubes is a good matrix for the lipase loading and immobilization. Typically, about 19.5%(wt) of the enzyme was successfully attached to the functionalized Mw-CNTs. The nano-composite material shows high thermal stability and good hydrophobic characteristics which considered important properties for the industrial implication. The immobilized enzyme shows acceptable activity and good stability in the medium operation conditions. The amount of the enzyme and the oxidation of the CNTs using the acids affects the efficiency of the immobilization severely. Beside that, other conditions like stirring rate, sonication of the Mw-CNTs, and the concentration of the cross linkers can also affect the process. Immobilized lipase on our Mw-CNTs could be used in the oily waste water pretreatment, hence it exhibits high degradation of the complex fatty acids in conditions similar to the real life industry.

CHAPTER 6

MICROWAVE PRETREATMENT FOR THE OILY WASTE WATER

Abstract

In this study, the effect of microwave (MW) pretreatment for emulsified waste water was studied. The effect of MW exposure time on the emulsion temperature and the degree of separation were analysed. Olive oil, gum Arabic, and amine acetate were used to simulate the waste water in the lab. Synthetic emulsions were placed in the microwave oven for different time intervals and left for sedimentation to see the effect of the MW in the degree of separation . Native and immobilized lipase were used to degrade and treat the samples after introduced to the microwave. Lipolysis reaction was done in optimum conditions as studied in chapter 2.4 and direct titration was used to calculate the activity of the enzyme.

6.1 Introduction

A primary hurdle in traditional lipids-rich waste water treatment is the formation of emulsions. Emulsions pose a serious problem for processing aqueous streams by any used method: mechanical, chemical and/or thermal. Additionally, they pose a vexing maintenance problem for treatment facilities due to their high affinity to depositing on pipe walls and fouling filtration media. Therefore, breaking the oil/water emulsion phases is a necessity for successful waste water treatment. The most important target of waste water treatment technologies is the removal of pollutants and organic compounds from the polluted water by different techniques and systems. There are many physical, chemical and biological methods used for efficient treatment and removal with suitable capacity. Beside the many advantage of novel waste water treatment technologies, such as advanced oxidation processes, membrane operations and biological treatment, sludge handling present actual solving problem for waste water treatment plant [138].

Various mechanical, ultrasonic, chemical, and thermal methods have been proposed to accelerate the hydrolysis of the waste water and sludge particles in anaerobic digestion. Although the thermochemical pretreatment of sludge results in an increased biodegradability by approximately 70%, the process consumes a substantial amount of energy as well as chemical consumption. Chemical treatments such as use of sodium hydroxide frequently create aggressive reaction condition and because of that, microwave is used as an alternative technique for the sludge and waste water pretreatment [17].

Microwave (MW) irradiation method has great advantage over the conventional thermal treatment. It is suitable to produce rapidly focused direct heat with low transmission energy losses. The existence of so called non thermal microwave effect are assumed arisen from the changing in dipole orientation of polar molecules, nuclear spin rotation and spin alignment manifested in restructuration of side chains and hydrogen bounds of macromolecules in high frequency alternating electromagnetic field. Kliala in 1983 and Wolf in 1986 were the first scientists suggest the idea of microwave emulsions treatment in their patent applications [48, 49]. Pretreatment of waste water via microwave (MW) irradiation is emerging as a promising alternative of traditional treatment methods: mechanical, chemical and thermal. Microwaves have proved to be an efficient alternative method, especially for thermal processes, for its inherent capability of delivering a focused direct heat with minimal energy losses. This direct and rapid heat result from the unique properties of electromagnetic waves in directing the energy to the water phase resulting with dipolar rotations and heat generation. In addition to, the obvious thermal advantages of MW energy, nonthermal advantages are also harnessed in the form disintegrating various particles in the treated media, as well as, in the form of disinfection via cell lysing of present microorganisms.

The main requirements for MW applications are the good degradation efficiency for various organic pollutants and rapid heating due to the high energy absorption. Because of the high water content MW operations can answer the challenge of fast heating and it can be made suitable for cost effective method with optimization of process parameters. Recently, active research and development work on microwave demul-

sification technology for applications in chemical plants was carried out by Samardzija [50]. Following his patent award, Klaila conducted several field tests, with a 50 kW microwave generator, which was equipped with wave guides and a microwave power monitor [48]. Fang et al in Kansas, used a large storage tank (ten ft in diameter and ten ft high) to treat 120 $bbls$ of slop oil. The emulsion was 50% oil, 27.5% water and 22.5% sediment. After applying 228 kWh of microwave energy continuously, the temperature of the top portion of emulsion reached approximately 100^oC and the emulsion was separated to oil and water layers [139]. Similar result was obtained in the field test in Louisiana for a tank contained 188 $bbls$ of crude oil water emulsion, but this time the tank had a height of 15 ft and 10 ft diameter. After exposing for 18.2 hours and using 417.5 kWh of radiation power with the emulsion, 146 barrels of oil was separated from 42 barrels of clear water [139]. In 2002 Camila et al studied the effect of microwaves on different oil water emulsion percentages. All cases shows that the separation degree of the water with the increase of the exposure time. They also studied the effect of $NaOH$ and they found that it presence in the emulsion lower water separation percentages. In another study they observed the initial ratio of the water to oil controls the maximum exposure time, hence, the maximum exposure time for 30% oil to 70% water emulsions is 300 seconds, while it was 240 seconds for 17% to 83% oil/water emulsions [140]. Vladana et al; studied the effect of microwave in line with freeze/thaw method in emulsion breaking and demulsification [53]. By varying the amount of oil from 0.1 to 30% in the emulsion and applying techniques above using microwave oven ($95^oC, 800\ W$, and 2450 MHZ). They found that applying

MW radiation made the molecules excited, this excitement resulted in super heating and high rate of reactions. The speed and the efficiency of the demulsification process was increased by the use of microwave radiation, and it is recommended as an efficient method, regarding the thawing technique used in their experiments. Finally, the efficiency for oil water emulsions up to 30% oil was tested to be above 90% [53]. Coutinho et al. patented a method of microwave emulsion treatment by optimizing many parameters such as water amount, content of salts, value of pH for the aqueous phase, initial temperature, microwave power, final temperature and drop size distribution to be adjusted in the industrial plant. Each 80 ml of the emulsion was exposed to 1400 W microwave power for 15 min and then followed by 10 min sedimentation time, the amount of water separated by the well-known Karl Fischer titration. After detailed study, the efficiency found to be around 25% of separated water and the ideal pH was found to be 7.0 to 9.5. The efficiency of the process is increasing proportionally with the increase of temperature above 90^oC up to 130^oC [54].

In this study, microwave pretreatment for two emulsions were studied to investigate the effect of the emulsion separation on the lipase enzyme hydrolysis of the oily waste water. Different exposure time to the microwave irradiation was applied for both samples. After that free and immobilized lipase on carbon nanotubes were used to perform the hydrolysis degradation of the oily synthetic waste water. It is expected that the microwave effect on the emulsion breaking will affect the degradation of oils separated from the emulsion.

6.2 Materials and Methodology

6.2.1 Materials

Candida Rugosa Lipase was purchased from Sigma Aldrich Co. US. Purified high quality olive oil was supplied locally from Hail Agricultural Development Co. KSA. Gum Arabic and amine acetate were supplied from Sigma Aldrich US. Absolute Ethanol, Hydrochloric Acid, Sodium Hydroxide phosphate buffer and other chemicals that have been used were supplied from local companies with laboratory grads. Domestic microwave device that has an ordinary range of microwaves $2.45GHz$. Deionized water was used as solvent for making solutions.

6.2.2 Olive oil/water emulsions preparation

To prepare the Olive oil/gum Arabic emulsion, typically, 10 g of olive oil and gum Arabic was added and mixed in a 400 ml beaker and the volume was brought to 200 ml using a 50 mM sodium phosphate buffer with pH 8.0, and homogenized for 5 min using a domestic blender. The emulsion was then contentiously stirred using a magnet stirrer during the daily use and a fresh emulsion was prepared in each time. Amine acetate salt was used as a surfactant to prepare another type of oil/water emulsion. $1\%(v/v)$ of amine acetate was added to 4% (v/v) pure olive oil in suitable beaker. This mixture was then heated in the electric oven for 10 min up to 70^oC until the salt completely molten and homogeneous mixture had been gotten. Hence, the melting point for the amine acetate salt is $(60\ to\ 80^oC)$. The mixture was taken out and immediately introduced to magnetic stirring followed by the addition of water 95%

(v/v). Tap water was added to the hot mixture gently drop by drop using glass funnel. This process ensures that the emulsion is gradually and steadily formed and at the end strong stable emulsion was obtained.

6.2.3 The effect of the microwave exposure time on lipase activity

Olive oil/gum Arabic emulsion was prepared and introduced to the microwave oven. Typically, 30 ml of emulsion was placed into a small flask and the increase of temperature was monitored as the exposure time increases. This step was important to investigate the heating rate and the temperature relation with the exposure time inside the MW oven. Temperature versus time curve was generated and therefore a clear approximation of the temperature inside the samples at any period of time was known. Microwave was used to break the emulsion and prepare the water sample for the hydrolysis reaction. Using a domestic microwave with a power of $900 - 1000$ w adjusted to (900 w) with different contacting time ranged from 60 to 180 S. The temperature generated form the microwave was monitored for different exposure times to predict the exact temperature while treating using the microwave.

The microwave pretreatment for three different samples with varied exposure time $(60, 120, and$ 180 Seconds) and for each sample the lipolysis reaction was performed and sub-samples of 4 ml had been extracted in different time intervals to determine the effect of microwave and the oil water separation on lipase activity. Each subsample then was quenched in absolute ethanol and titrated with NaOH to determine

the amount of liberated fatty acids. For the olive oil/gum Arabic emulsion, the oil concentration was about 0.1 g/ml and the enzyme amount was 1 mg/ml. Adjusting the pH to 8 using potassium phosphate and the temperature was controlled to be fixed at 30^oC using a hot bath. The microwave radiation exposure time was then changed from 60 to 180 S and for each exposure time sample, constantly, 4 ml sub-samples has been extracted from the reaction vessel. Hence, the reaction time was varied from 0.5 to 24 hr, keeping the mixing speed constant as 300 rpm. Finally, 0.05 N NaOH had been used to titrate the subsamples after quenching with ethanol. For the second emulsion, olive oil/amine acetate the emulsion was prepared as mentioned above. Three samples were taken and different microwave exposure time was applied to the samples starting from 1, 2, and 3 min. The same steps followed with the first emulsion were conducted to measure the effect of the microwave emulsion treatment on the enzyme activity.

6.3 Results and discussion

6.3.1 The effect of microwave on emulsion separation

Figure 6.1 shows that the temperature of the emulsion increased linearly until the third minute where the temperature leveled off at nearly 95^oC where the sample started to boil. The maximum heating temperature that could be reached for the emulsified sample is about 99^oC nearly after 8 min heating time.

As reported by many previous studies in the literature [53, 54, 139, 141, 142], mi-

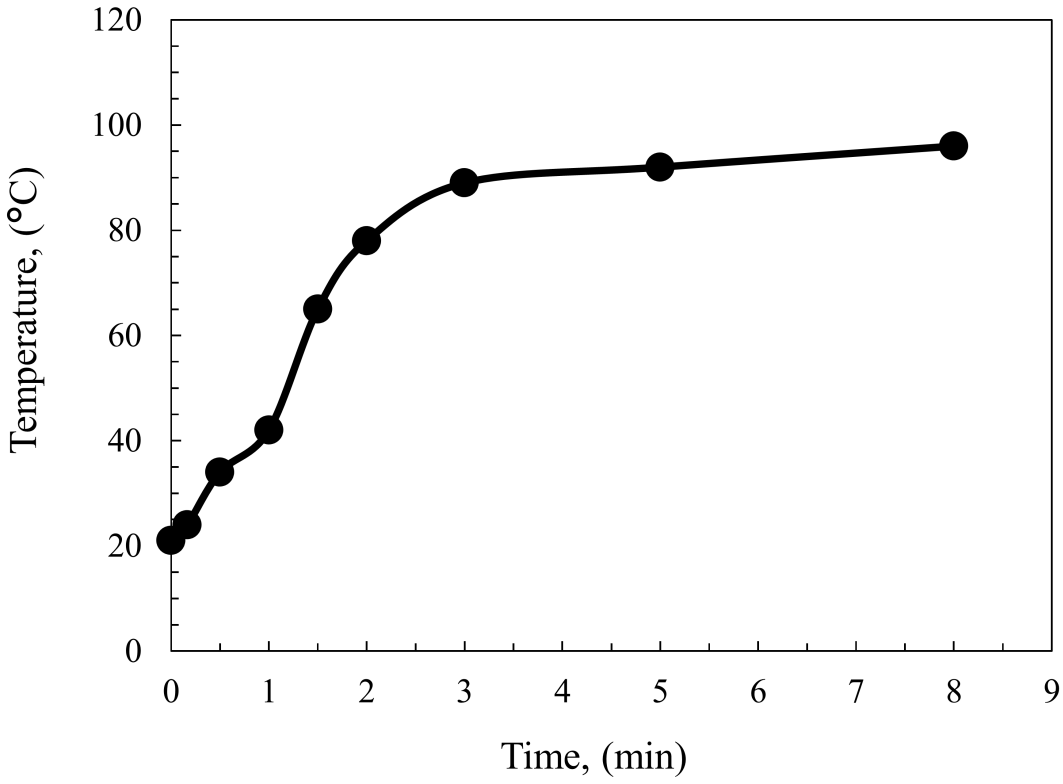

Figure 6.1: The effect of the exposure time on the emulsion temperature, olive oil/gum Arabic emulsion, 30 ml, frequency = 2.45 GHz (Domestic)

crowaves have clear effect on the separation of the emulsions. figure 6.2 represents three samples of olive oil/amine acetate emulsion before the pretreatment with microwave irradiation. The samples were placed in the microwave at different exposure time which was changed from $1, 2, and\ 3\ min$ for the samples figure 6.2.(a), figure 6.2.(b), and figure 6.2.(c), respectively. Comparing the same samples with figure 6.2 it was noticed that, exposing the emulsion to the microwave for 3 min totally separated the emulsion into oil and water layers and decreased the exposure time decreases the separation. Hence, 1 min is not enough for the microwaves to disturb the electric layer of the emulsion molecules.

6.3.2 The effect of emulsion separation on the hydrolysis reaction

As shown in figure 6.3, olive oil/gum Arabic emulsion shows no effect when pretreated with microwaves. The rate of reaction remains almost the same for the three samples when introduced to different exposure time from 1, 2, and 3 min, to the microwaves. Due to the polymeric nature of the gum Arabic, hence it contains polysaccharide, protein and arabino galacto protein species, the steric and electrostatic stabilization of the emulsion molecules is strong [143]. The microwaves have no effect on the separation of such emulsions. Consequently, the rate of reaction remains the same, because the samples did not changed.

Figure 6.4 represents the activity of immobilized lipase on carbon nanotubes in the pretreated samples. It is clearly shown that free lipase is affected by the emulsion

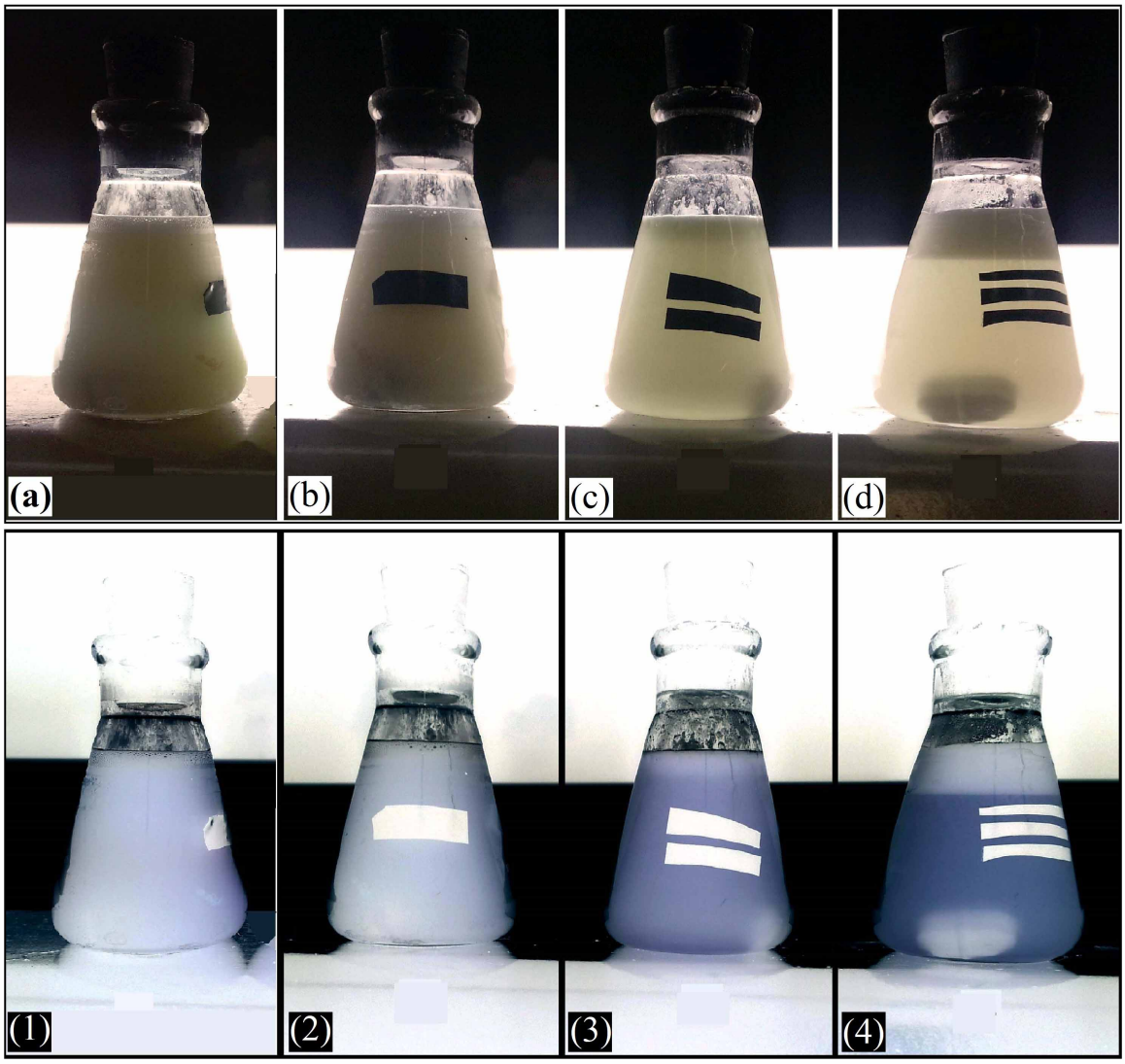

Figure 6.2: Effect of MW pretreatment of Olive oil/Amine acetate emulsion. Samples (a) and (1) is before the exposure, and after exposure to the microwave irradiation: (b) or (2) exposed for 1 *min*, (c) or (3) for 2 *min*, and (d) or(3) for 3 *min*

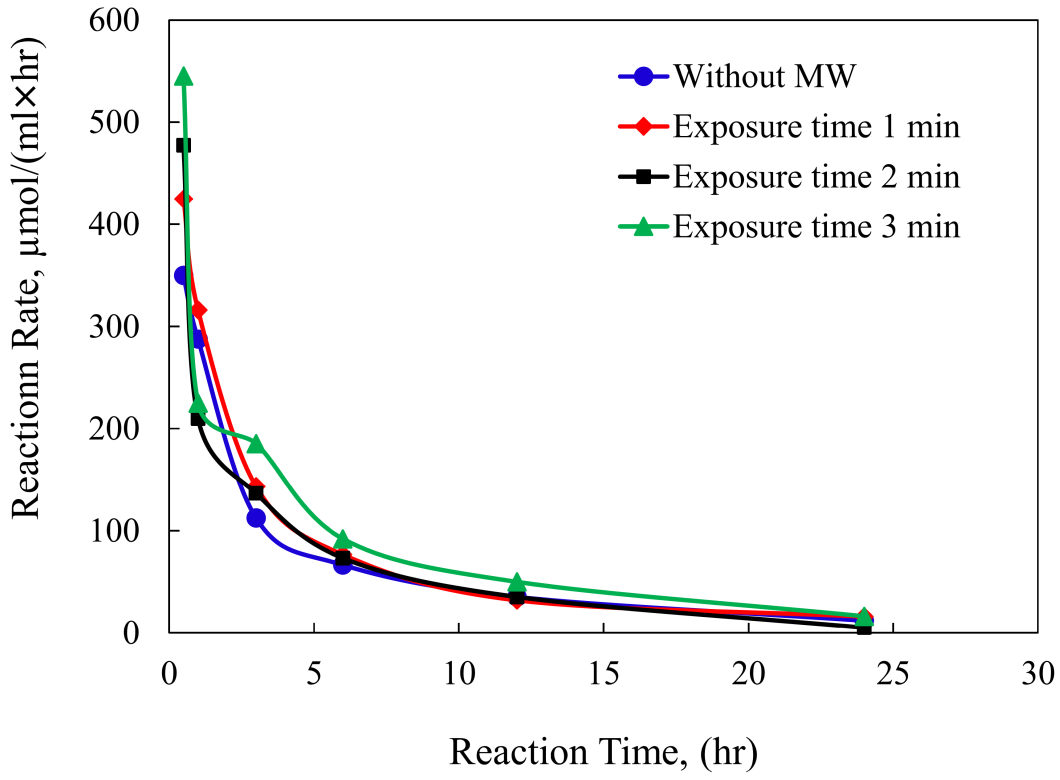

Figure 6.3: Shows the effect of emulsions different exposure times to microwave in the rate of the hydrolysis reaction using free lipase, olive oil/gum Arabic emulsion, reaction temperature = 37^oC, pH = 8, reaction time = 30 min, mixing speed = 200 rpm

separation specially when the samples were introduced to the microwave for long times. Exposing the samples to the MW for $3min$ increased the activity of the lipase severely as shown if figure 6.4. Hence, at this large exposure time the temperature reaches about 90^oC as shown in figure 6.1, the oil is completely separated from the water. This introduces more free water for the immobilized lipase to increase it is activity. It is well known that the lipase is a water soluble enzyme and it is activity increases rapidly with the availability of the water [56].

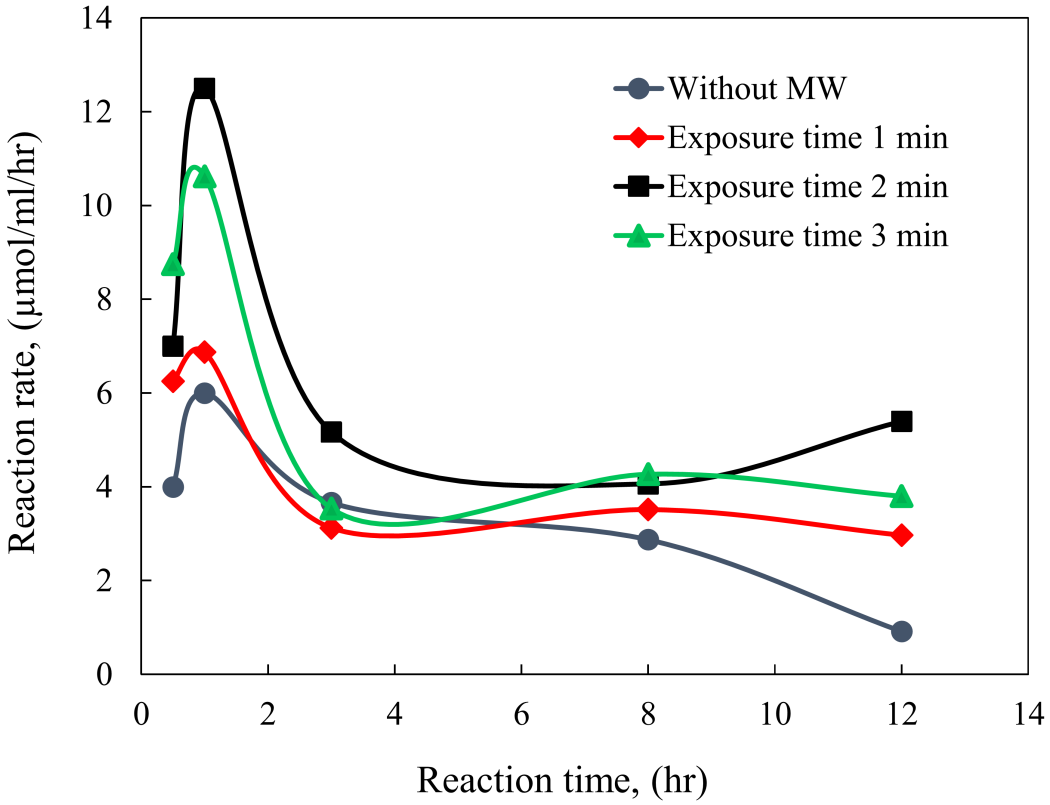

Figure 6.4: Effect of emulsions different exposure times to microwaves on the rate of hydrolysis reaction using free lipase, olive oil/amine acetate emulsion, reaction temperature = 37^oC, pH = 8, reaction time = 30 min, mixing speed = 200 rpm

Results in figure 6.5 shows that the microwave pretreatment has no effect on the

MwCNT-lipase activity and the reaction rate. Although the results from figure 6.2 shows that there is good separation for the olive oil/amine acetate emulsion, yet there is no change in the hydrolysis reaction. However, slight variation in the values at the early hours of the reaction and it may refer to the instability of the conditions at the beginning of the experiment.

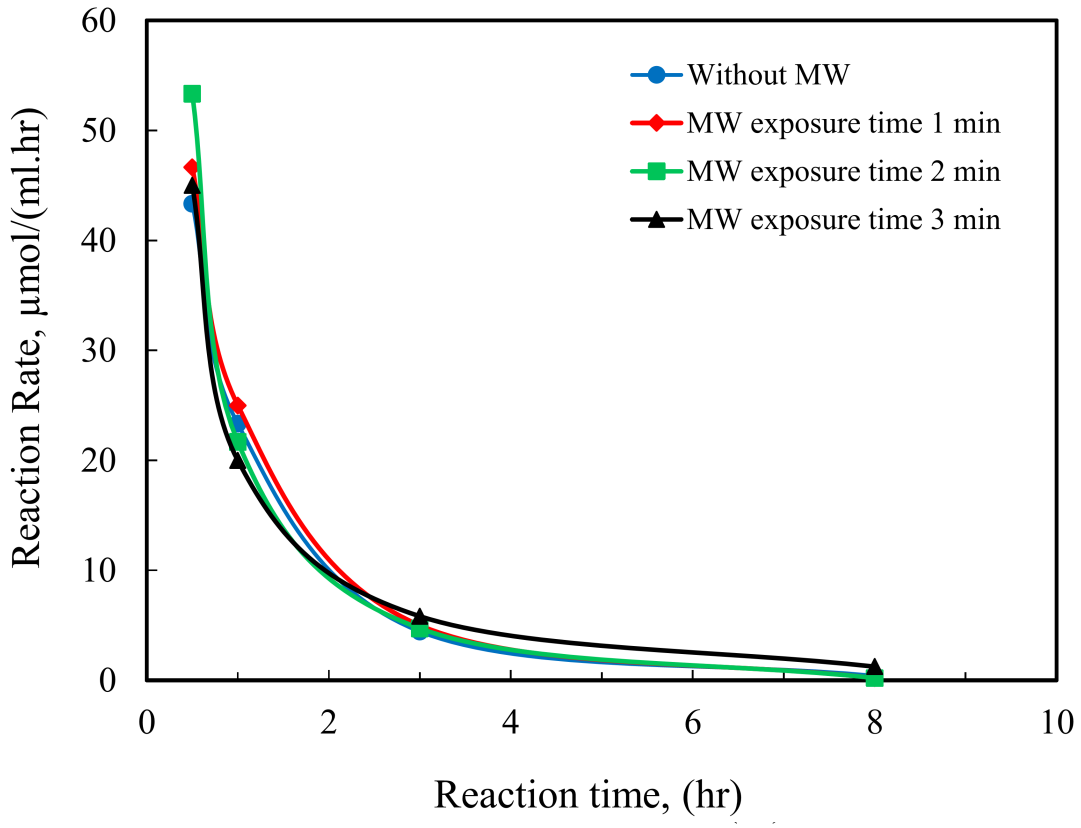

Figure 6.5: Shows the effect of emulsions different exposure times to microwave in the rate of the hydrolysis reaction using MwCNT-Lipase, olive oil/amine acetate emulsion, reaction temperature = 37^oC, pH = 8, reaction time = 30 min, mixing speed = 200 rpm

6.4 Conclusion

The effect of the microwave pretreatment on the oil/water emulsion stability and separation was studied and investigated by exposing oil emulsion samples to MW irradiation. Two different emulsions were prepared and had been investigated for this purpose. Olive oil/gum Arabic emulsion shows high stability when exposed to the microwaves irradiation and the addition of free lipase to check the effect of the pretreatment on the hydrolysis of the oils confirmed the result that no separation had happened. The olive oil/amine acetate emulsion was also studied for the pretreatment using the microwaves. It showed high degree of separation when exposed to the microwave for long time. As the temperature of the emulsion increases, the degree of separation increases. The separation had great effect on the hydrolysis of the olive oil, specially when the free lipase is used. However, no significant effect noticed on the immobilized lipase samples.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

In this study, a comprehensive investigation of the enzymatic treatment of oily waste water assisted by microwave was performed. Lipase enzyme was carefully studied for oily emulsions hydrolysis and multi-wall carbon nanotubes which had been synthesized locally was used as a support material for lipase immobilization. Synthetic oily waste water was prepared in the lab to simulate the real waste water. Microwave pretreatment applied to the synthetic emulsions and the effect of microwaves on its stability and separation of oil-water phases was investigated.

Olive oil emulsions was used to investigate the kinetics and hydrolysis reactions catalyzed by lipase enzyme. Reaction parameters such as temperature, pH, mixing speed, and enzyme concentration were studied. After a detailed investigation, best reaction conditions for the olive oil hydrolysis was found to be 35^oC, pH of 8, and 0.7

g (enzyme)/ml (emulsion). A mathematical kinetics model was derived for the reaction and it showed good agreement with the experimental data obtained. Both linear (Hanes-Woolf model) and non-linear (Mathematica) fitting were used to estimate the cofficients for proposed model. Although both of them had shown excellent approximation, yet the last one is more accurate and had the closest results. The model that had been obtained was in a good agreement with what stated in the literature.

Floating head chemical vapor deposition reactor was used to synthesize local multi-wall carbon nano-tubes. SEM and TGA analysis showed that the produced CNTs had good properties including length, purity, and aspect ratio. Using nitric acid oxidation, the produced Mw-CNT was purified and treated from catalyst impurities and amorphous carbon attached to the tube bundles. FTIR analysis confirmed the appearance of carboxyl groups bands in the oxidized CNTs. The treated samples shows purity up to 97% (wt) and 10% (wt) of carboxyl groups when analyzed using TGA. With this properties, Mw-CNTs produced locally was used as a support material for lipase immobilization.

Covalent binding between the treated Mw-CNTs and lipase enzyme was obtained using organic cross-linkers (NHS and EDC). TGA results for the functionalized samples (MwCNT-Lipase) showed that 19.5% (wt) of the enzyme was successfully attached to the produced Mw-CNTs. The nano-composite material showed high reaction activity and good hydrophobic characteristics which considered important properties for the industrial implication. Immobilized lipase, with this unique properties, was used with microwave pretreatment to hydrolyse the oily emulsions prepared locally.

Microwave irradiation was applied to locally prepared oil/water emulsions and the degree of emulsion separation was investigated. Olive oil/gum arabic emulsion showed high stability when exposed to the microwaves irradiation up to boiling temperatures. However, olive oil/ammonium acetate emulsion showed high degree of separation when exposed to the microwave for 3 min (96^oC). Emulsion separation had great effect on olive oil hydrolysis when free lipase was used after microwave pretreatment. Nevertheless, no significant effect was noticed when immobilized lipases were used. This results confirmed the unique properties of the immobilized lipase synthesized.

Results that we obtained confirms that the immobilized lipase that synthesized locally could be used as an efficient material for oily pollutants removal from waste water.

7.2 Recommendations

By finishing this study we recommend:

To investigate the effect of the assisted microwave enzymatic treatment on real waste water samples with different oil concentrations and study the effect of separation of emulsions on oil removal in wider range of reaction conditions.

To apply the immobilized lipase on produced waste water coming from oil industry, since it makes allot of environmental issues, and study the degradation of complex heavy oils using such environmentally friendly materials and methods.

To use other materials as support matrices such as graphene, carbon nano-fibers, and other carbon structured nano-materials and investigate its effect on enhancing the properties of the enzyme.

REFERENCES

[1] P. F. Rupani, R. P. Singh, M. H. Ibrahim, and N. Esa, "Review of Current Palm Oil Mill Effluent (POME) Treatment Methods : Vermicomposting as a Sustainable Practice," vol. 10, no. 10, pp. 1190–1201, 2010.

[2] N. G. Wakelin and C. F. Forster, "An investigation into microbial removal of fats, oils and greases," *Bioresource Technology*, vol. 59, no. 1, pp. 37–43, Jan. 1997.

[3] T. V. Adulkar and V. K. Rathod, "Ultrasound assisted enzymatic pre-treatment of high fat content dairy wastewater," *Ultrasonics Sonochemistry*, vol. 21, no. 3, pp. 1083–1089, 2014.

[4] M. Cheryan and N. Rajagopalan, "Membrane processing of oily streams. Wastewater treatment and waste reduction," *Journal of Membrane Science*, vol. 151, no. 1, pp. 13–28, Dec. 1998.

[5] J. Coca-Prados and G. Gutiérrez-Cervelló, *Water Purification and Management*, 2011.

[6] X. Lefebvre, E. Paul, M. Mauret, P. Baptiste, and B. Capdeville, "Kinetic characterization of saponified domestic lipid residues aerobic biodegradation," *Water Research*, vol. 32, no. 10, 1998.

[7] R. Petruy and G. Lettinga, "DIGESTI0N 0F A M1LK FAT EMULSI0N," *Bioresource technology*, vol. 61, no. 1988, pp. 141–149, 1997.

[8] J. B. Williams, C. Clarkson, C. Mant, A. Drinkwater, and E. May, "Fat, oil and grease deposits in sewers: characterisation of deposits and formation mechanisms," *Water research*, vol. 46, no. 19, pp. 6319–6328, Dec. 2012.

[9] B. Brena, P. González-Pombo, and F. Batista-Viera, "Immobilization of enzymes: a literature survey." *Methods in molecular biology (Clifton, N.J.)*, vol. 1051, pp. 15–31, 2013.

[10] I. V. Pavlidis and T. Tsoufis, "Functionalized MultiWall Carbon Nanotubes for Lipase Immobilization," *Advanced Biomaterials*, vol. 1, no. 5, pp. 179–183, 2010.

[11] H. R. Luckarift, G. R. Johnson, and J. C. Spain, "Silica-immobilized enzyme reactors; application to cholinesterase-inhibition studies." *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, vol. 843, no. 2, pp. 310–6, Nov. 2006.

[12] X.-J. Huang, A.-G. Yu, and Z.-K. Xu, "Covalent immobilization of lipase from Candida rugosa onto poly(acrylonitrile-co-2-hydroxyethyl methacrylate) electrospun fibrous membranes for potential bioreactor application." *Bioresource technology*, vol. 99, no. 13, pp. 5459–5465, Sep. 2008.

[13] R. A. Sheldon, "Enzyme Immobilization: The Quest for Optimum Performance," *Advanced Synthesis & Catalysis*, vol. 349, no. 8-9, pp. 1289–1307, Jun. 2007.

[14] J. Kim, J. W. Grate, and P. Wang, "Nanostructures for enzyme stabilization," *Chemical Engineering Science*, vol. 61, no. 3, pp. 1017–1026, Feb. 2006.

[15] J. Kim, J. Grate, and P. Wang, "Nanobiocatalysis and its potential applications," *Trends in biotechnology*, vol. 26, no. 11, pp. 639–646, 2008.

[16] N. M. D.A. Jones, T.P. Lelyveld, S.D. Mavrofidis, S.W. Kingman, "Microwave heating applications in environmental engineering: a review," *Resources, conservation and recycling*, vol. 34, pp. 75–90, 2002.

[17] J. Osepchuk, "A history of microwave heating applications," *Microwave Theory and Techniques, IEEE*, vol. MTT-32, no. 9, p. 1200 1224, 1984.

[18] R. Kelly and N. Rowson, "Microwave reduction of oxidised ilmenite concentrates," *Minerals Engineering*, vol. 8, no. 11, pp. 1427–1438, 1995.

[19] J. Hill and T. Marchant, "Modelling microwave heating," *Applied mathematical modelling*, vol. 20, no. 1, pp. 3–15, Jan. 1996.

[20] C. Reimbert and A. A. Minzoni, "Effect of radiation losses on hotspot formation and propagation in microwave heating," *IMA Journal of Applied Mathematics*, vol. 57, pp. 165–179, 1996.

[21] G. F. Bennett and R. W. Peters, "The removal of oil from wastewater by air flotation: A review," *Critical Reviews in Environmental Control*, vol. 18, no. 3, pp. 189–253, Jan. 1988.

[22] J. Rubio, M. L. Souza, and R. W. Smith, "Overview of flotation as a wastewater treatment technique," *Minerals Engineering*, vol. 15, no. 3, pp. 139–155, Mar. 2002.

[23] N. D. Sylvester and J. J. Byeseda, "Oil/water separation by induced-air flotation," *Society of Petroleum Engineers Journal*, no. December, 1980.

[24] P. Paraskeva and E. Diamadopoulos, "Technologies for olive mill wastewater (OMW) treatment: a review," *Journal of Chemical Technology & Biotechnology*, vol. 1485, no. December 2005, pp. 1475–1485, 2006.

[25] T. N. Aziz, L. M. Holt, K. M. Keener, J. W. Groninger, and J. J. Ducoste, "Performance of Grease Abatement Devices for Removal of Fat, Oil, and Grease," *Journal of Environmental Engineering*, vol. 137, no. 1, pp. 84–92, Jan. 2011.

[26] A. Fakhru'l-Razi and A. Pendashteh, "Review of technologies for oil and gas produced water treatment," *Journal of Hazardous Materials...*, vol. 170, pp. 530–551, 2009.

[27] P. Chowdhury, T. Viraraghavan, and A. Srinivasan, "Biological treatment processes for fish processing wastewater–a review." *Bioresource technology*, vol. 101, no. 2, pp. 439–449, Jan. 2010.

[28] B. Demirel, O. Yenigun, and T. T. Onay, "Anaerobic treatment of dairy wastewaters: a review," *Process Biochemistry*, vol. 40, no. 8, pp. 2583–2595, Jul. 2005.

[29] J. Rajesh Banu, S. Anandan, S. Kaliappan, and I.-T. Yeom, "Treatment of dairy wastewater using anaerobic and solar photocatalytic methods," *Solar Energy*, vol. 82, no. 9, pp. 812–819, Sep. 2008.

[30] V. Alexandre, a.M. Valente, M. C. Cammarota, and D. M. Freire, "Performance of anaerobic bioreactor treating fish-processing plant wastewater pre-hydrolyzed with a solid enzyme pool," *Renewable Energy*, vol. 36, no. 12, pp. 3439–3444, Dec. 2011.

[31] C. Kalyanaraman, K. Sri Bala Kameswari, V. Sudharsan Varma, S. Tagra, and J. Raghava Rao, "Studies on biodegradation of vegetable-based fat liquor-containing wastewater from tanneries," *Clean Technologies and Environmental Policy*, vol. 15, no. 4, pp. 633–642, Dec. 2012.

[32] Alade A. O., Jameel A.T., Muyubi S. A., Abdul Karim M. I. and A. M. Z., "REMOVAL OF OIL AND GREASE AS EMERGING POLLUTANTS OF CONCERN (EPC) IN WASTEWATER STREAM," *IIUM Engineering Journal*, vol. 12, no. 4, pp. 161–169, 2011.

[33] T. Godfrey, "Introduction to industrial enzymology, Godfrey T., West S., Industrial enzymology, 1996, 1-8," *Stockholm Press, New York*.

[34] R. Sharma, Y. Chisti, and U. Banerjee, "Production, purification, characterization, and applications of lipases," *Biotechnology advances*, vol. 19, pp. 627–662, 2001.

[35] A. Jorio, R. Saito, J. Hafner, C. Lieber, M. Hunter, T. McClure, G. Dresselhaus, and M. Dresselhaus, "Structural (n, m) Determination of Isolated Single-Wall Carbon Nanotubes by Resonant Raman Scattering," *Physical Review Letters*, vol. 86, no. 6, pp. 1118–1121, Feb. 2001.

[36] R. Saito, G. Dresselhaus, and M. Dresselhaus, *Physical properties of carbon nanotubes*, 1998.

[37] T. W. Ebbesen, *Carbon Nanotubes: Preparation and Properties*. CRC Press, 1996.

[38] S. Iijima, "Helical microtubules of graphitic carbon," pp. 56–58, 1991.

[39] T. Ebbesen and P. Ajayan, "Large-scale synthesis of carbon nanotubes," *Nature*, 1992.

[40] Sumio Iijima and Toshinari Ichihashi, "Single shell carbon nanotubes of 1-nm diameter," *Nature*, vol. 363, 1993.

[41] S. Tans, M. Devoret, H. Dai, and A. Thess, "Individual single-wall carbon nanotubes as quantum wires," *letters to Nature*, 1997.

[42] M. JoseÌ-YacamaÌn, M. Miki-Yoshida, L. RendoÌn, and J. G. Santiesteban, "Catalytic growth of carbon microtubules with fullerene structure," *Applied Physics Letters*, vol. 62, no. 2, p. 202, 1993.

[43] W. Tischer and F. Wedekind, "Immobilized enzymes: methods and applications," *Biocatalysis-from discovery to application*, vol. 200, 1999.

[44] C. M. Soares, H. F. De Castro, F. F. De Moraes, and G. M. Zanin, "Characterization and utilization of Candida rugosa lipase immobilized on controlled pore silica." *Applied biochemistry and biotechnology*, vol. 77-79, pp. 745–757, 1999.

[45] H.-K. Lee, J.-K. Lee, M.-J. Kim, and C.-J. Lee, "Immobilization of Lipase on Single Walled Carbon Nanotubes in Ionic Liquid," *Bulletin of the Korean Chemical Society*, vol. 31, no. 3, pp. 650–652, Mar. 2010.

[46] S. G. Wang, Q. Zhang, R. Wang, S. F. Yoon, J. Ahn, D. J. Yang, J. Z. Tian, J. Q. Li, and Q. Zhou, "Multi-walled carbon nanotubes for the immobilization of enzyme in glucose biosensors," *Electrochemistry Communications*, vol. 5, no. 9, pp. 800–803, Sep. 2003.

[47] P. Ji, "Lipase Covalently Attached to Multiwalled Carbon Nanotubes as an Efficient Catalyst in Organic Solvent," *AIChE Journal*, vol. 56, no. 11, pp. 3005–3011, 2010.

[48] W. J. Klaila and F. T. Sullivan, "METHOD AND APPARATUS FOR CONTROLLING FLUENCY OF HIGH VISCOSITY HYDROCARBON FLUIDS," *US Patent 4,067,683*, 1978.

[49] Nicholas 0. Wolf, "USE OF MICROWAVE RADIATION IN SEPARATING EMULSIONS AND DISPERSIONS OF HYDROCARBONS AND WATER," *United States Patent*, 1986.

[50] N. Samardzija, "Apparatus for microwave separation of emulsions," 1989.

[51] C. Fang and P. Lai, "Microwave heating and separation of water-in-oil emulsions," *Journal of microwave power and electromagnetic Energy*, 1995. [Online]. Available: http://www.jmpee.org/JMPEE_PDFs/30-1/JMPEE_Microwave_Heating_and_Separation_of_Water-in-Oil_Emulsions.pdf

[52] V. Camila and D. Mayolett, "Treatment of waste-water/oil emulsions using microwave radiation," *Proceedings of SPE International Conference on Health, Safety and Environment in Oil and Gas Exploration and Production*, Mar. 2002.

[53] V. Rajaković and D. Skala, "Separation of water-in-oil emulsions by freeze/thaw method and microwave radiation," *Separation and Purification Technology*, vol. 49, pp. 192–196, 2006.

[54] R. Coutinho and M. Heredia, "Method for the microwave treatment of water-in-oil emulsions," *United States Patent*, 2010.

[55] M. Arbige and W. Pitcher, "Industrial enzymology: a look towards the future," *Trends in Biotechnology*, 1989.

[56] S. Al-Zuhair, M. Hasan, and K. B. Ramachandran, "Kinetics of the enzymatic hydrolysis of palm oil by lipase," *Process Biochemistry*, 2003.

[57] S. W. Tsai, G. H. Wu, and C. L. Chiang, "Kinetics of enzymatic hydrolysis of olive oil in biphasic organic-aqueous systems." *Biotechnology and bioengineering*, vol. 38, no. 7, pp. 761–6, Oct. 1991.

[58] T. Kuo and H. Gardner, *Lipid biotechnology*. Marcel Dekker, Inc, 2002.

[59] S. Facchin, "Biodiversity and secretion of enzymes with potential utility in wastewater treatment," *Open Journal of Ecology*, vol. 03, no. 1, pp. 34–37, 2013.

[60] P. Kuhl, "Excess-substrate inhibition in enzymology and high-dose inhibition in pharmacology: a reinterpretation," *Biochem. J*, vol. 180, pp. 171–180, 1994.

[61] Praphan Pinsirodom and Kirk L. Parkin, "Current Protocols in Food Analytical Chemistry_ Lipase Assays," in *Current Protocols in Food Analytical Chemistry*, 2001, pp. 1–13.

[62] F. Beisson and A. Tiss, "Methods for lipase detection and assay: a critical review," *European Journal of Lipid ...*, 2000.

[63] F. Hasan, A. A. Shah, and A. Hameed, "Industrial applications of microbial lipases," *Enzyme and Microbial Technology*, vol. 39, no. 2, pp. 235–251, Jun. 2006.

[64] M. Martinelle, M. Holmquist, and K. Hult, "On the interfacial activation of< i> Candida antarctica</i> lipase A and B as compared with< i> Humicola lanuginosa</i> lipase," *Biochimica et Biophysica Acta (BBA)- ...*, 1995.

[65] Glycerine, *Glycerine: A Key Cosmetic Ingredient*. CRC Press, 1991.

[66] M. Canakci and J. V. Gerpen, "Biodiesel production from oils and fats with high free fatty acids," *Transactions of the ASAE*, vol. 44, no. 6, pp. 1429–1436, 2001.

[67] A. Kumar Tiwari, A. Kumar, and H. Raheman, "Biodiesel production from jatropha oil (Jatropha curcas) with high free fatty acids: An optimized process," *Biomass and Bioenergy*, vol. 31, no. 8, pp. 569–575, Aug. 2007.

[68] S.-W. Tsai and C.-S. Chang, "Kinetics of lipase-catalyzed hydrolysis of lipids in biphasic organic-aqueous systems," *Journal of Chemical Technology & Biotechnology*, vol. 57, no. 2, pp. 147–154, Apr. 1993.

[69] S. Mukataka and T. Kobayashi, "Enzymatic hydrolysis of fats at high substrate concentrations in biphasic organic-aqueous systems," *Journal of fermentation ...*, vol. 65, no. 1, pp. 23–29, 1987.

[70] G. Pierce, C. Wick, and D. Palmer, "Unique Microbial lipases with activity at temperatures and pHs suitable for use in detergents." 1990.

[71] P. Calderbank, "Physical rate processes in industrial fermentation. Part I: The interfacial area in gas-liquid contacting with mechanical agitation," *Trans. Inst. Chem. Eng*, 1958.

[72] C. Albasi, N. Bertrand, and J.-P. Riba, "Enzymatic hydrolysis of sunflower oil in a standardized agitated tank reactor," *Bioprocess Engineering*, vol. 20, no. 1, p. 77, 1999.

[73] S. Al-Zuhair, K. B. Ramachandran, and M. Hasan, "Investigation of the specific interfacial area of a palm oilâ€"water system," *Journal of Chemical Technology & Biotechnology*, vol. 79, no. 7, pp. 706–710, Jul. 2004.

[74] A. Krakowiak, M. TrzciÆ'ska, B. Sieliwanowicz, and R. Sawicka, "Properties of immobilized and free lipase from," *POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES*, vol. 12, no. 3, pp. 39–44, 2003.

[75] Praphan Pinsirodom and Kirk L. Parkin, "Lipase Assays," *Current Protocols in Food Analytical Chemistry*, vol. C3.1, pp. 1–13, 2001.

[76] S. T. Kang and J. S. Rhee, "Characteristics of immobilized lipase-catalyzed hydrolysis of olive oil of high concentration in reverse phase system." *Biotechnology and bioengineering*, vol. 33, no. 11, pp. 1469–76, May 1989.

[77] S. Saktaweewong, P. Phinyocheep, C. Ulmer, E. Marie, A. Durand, and P. Inprakhon, "Lipase activity in biphasic media: Why interfacial area is a significant parameter?" *Journal of Molecular Catalysis B: Enzymatic*, vol. 70, no. 1-2, pp. 8–16, Jun. 2011.

[78] S. Gupta, P. Ingole, K. Singh, and A. Bhattacharya, "Comparative Study of the Hydrolysis of Different Oils by Lipase-Immobilized Membranes," 2011.

[79] C. J. Fielding, "Lipoprotein lipase: evidence for high- and low-affinity enzyme sites." *Biochemistry*, vol. 15, no. 4, pp. 879–84, Feb. 1976.

[80] Haldane, "The affinity of different types of enzymes for thier substrates," *Nature*, vol. 121, no. 3041, p. 207, 1928.

[81] R. Ritchie and T. Prvan, "Current statistical methods for estimating the Km and Vmax of Michaelisâ€Menten kinetics," *Biochemical Education*, vol. 2, no. V, pp. 196–206, 1996.

[82] S. Al-zuhair, "Kinetics of hydrolysis of tributyrin by lipase," *Journal of Engineering science and Technology*, vol. 1, no. 1, pp. 50–58, 2006.

[83] G. L. A. Nimmo and I. A., "A Comparision of Seven Methods for Fitting the Michaelis Menton Equation," *Biochemistry Journal*, vol. 216, no. Pt 10, p. 1757, May 1975.

[84] E. Jurado, F. Camacho, G. Luzón, M. Fernández-Serrano, and M. García-Román, "Kinetics of the enzymatic hydrolysis of triglycerides in o/w emulsions," *Biochemical Engineering Journal*, vol. 40, no. 3, pp. 473–484, Jul. 2008.

[85] S. Nagai, J. Bailey, and D. Ollis, "Biochemical Engineering Fundamentals," *. . . Engineering Fundamentals*, 1986.

[86] J. Bailey and D. Ollis, "Biochemical engineering fundamentals," 1986.

[87] M. Sémériva and P. Desnuelle, "Pancreatic lipase and colipase. An example of heterogeneous biocatalysis." *Advances in enzymology and related areas of molecular biology*, vol. 48, pp. 319–70, Jan. 1979.

[88] R. Saito, G. Dresselhaus, and M. Dresselhaus, "Physical properties of carbon nanotubes, 1998," *Imperial College, London*, 1998.

[89] M. Joseâ€Yacaman, "Catalytic growth of carbon microtubules with fullerene structure," *Applied Physics . . .*, 1993.

[90] D. Srivastava, K. Cho, and C. Wei, "Nanomechanics of carbon nanotubes and composites," *Applied . . .*, 2003.

[91] N. Prlainovic, D. Bezbradica, Z. Knezevic-Jugovic, A. Marinkovic, and D. Mijin, "Immobilization of enzymes onto carbon nanotubes," *Hemijska industrija*, vol. 65, no. 4, pp. 423–430, 2011.

[92] Q. Shi, D. Yang, Y. Su, J. Li, Z. Jiang, Y. Jiang, and W. Yuan, "Covalent functionalization of multi-walled carbon nanotubes by lipase," pp. 1205–1210, 2007.

[93] Y. Gao and I. Kyratzis, "Covalent Immobilization of Proteins on Carbon Nanotubes Using the," vol. 19, no. 10, 2008.

[94] M. Ghosh, S. Maiti, S. Dutta, D. Das, and P. K. Das, "Covalently functionalized single-walled carbon nanotubes at reverse micellar interface: a strategy to improve lipase activity." *Langmuir : the ACS journal of surfaces and colloids*, vol. 28, no. 3, pp. 1715–24, Jan. 2012.

[95] N. Saifuddin, a. Z. Raziah, and a. R. Junizah, "Carbon Nanotubes: A Review on Structure and Their Interaction with Proteins," *Journal of Chemistry*, vol. 2013, pp. 1–18, 2013.

[96] C. Lee, J. Park, Y. Huh, and J. Y. Lee, "Temperature effect on the growth of carbon nanotubes using thermal chemical vapor deposition," *Chemical physics letters*, vol. 343, no. July, pp. 33–38, 2001.

[97] S. Maruyama, R. Kojima, and Y. Miyauchi, "Low-temperature synthesis of high-purity single-walled carbon nanotubes from alcohol," *Chemical physics letters*, vol. 360, no. July, pp. 229–234, 2002.

[98] R. Brukh and S. Mitra, "Kinetics of carbon nanotube oxidation," no. 1, pp. 619–623, 2007.

[99] P. Serp, A. Madroñero, and J. Figueiredo, "Production of vapour-grown carbon fibres: influence of the catalyst precursor and operating conditions," *Fuel*, vol. 78, pp. 837–844, 1999.

[100] Y. Li, S. Bae, a. Sakoda, and M. Suzuki, "Formation of vapor grown carbon fibers with sulfuric catalyst precursors and nitrogen as carrier gas," *Carbon*, vol. 39, no. 1, pp. 91–100, Jan. 2001.

[101] L. Sun, Z. Liu, X. Ma, and D. Tang, "Growth of carbon nanofibers array under magnetic force by chemical vapor deposition," *Chemical physics ...*, vol. 336, no. March, pp. 392–396, 2001.

[102] M. Hussien, "Synthesis, Characterization and Application of Carbon Nanotubes and Carbon Nanofibers," 2005.

[103] J. Cheng, X. Zhang, F. Liu, J. Tu, H. Lu, Y. Sun, and F. Chen, "Long bundles of aligned carbon nanofibers obtained by vertical floating catalyst method," *Materials Chemistry and Physics*, vol. 87, no. 2-3, pp. 241–245, Oct. 2004.

[104] M. AlSaadi and A. Al-Mamun, "Synthesis of various carbon nanomaterials (CNMs) on powdered activated carbon," *African Journal of . . .*, pp. 1–25, 2011.

[105] M. Yudasaka, R. Kikuchi, T. Matsui, Y. Ohki, S. Yoshimura, and E. Ota, "Specific conditions for Ni catalyzed carbon nanotube growth by chemical vapor deposition," *Applied Physics Letters*, vol. 67, no. 17, p. 2477, 1995.

[106] Z. F. Ren, Z. P. Huang, D. Z. Wang, J. G. Wen, J. W. Xu, J. H. Wang, L. E. Calvet, J. Chen, J. F. Klemic, and M. a. Reed, "Growth of a single freestanding multiwall carbon nanotube on each nanonickel dot," *Applied Physics Letters*, vol. 75, no. 8, p. 1086, 1999.

[107] V. Datsyuk, M. Kalyva, K. Papagelis, J. Parthenios, D. Tasis, A. Siokou, I. Kallitsis, and C. Galiotis, "Chemical oxidation of multiwalled carbon nanotubes," vol. 6, pp. 2–9, 2008.

[108] M. W. Marshall, S. Popa-Nita, and J. G. Shapter, "Measurement of functionalised carbon nanotube carboxylic acid groups using a simple chemical process," *Carbon*, vol. 44, no. 7, pp. 1137–1141, Jun. 2006.

[109] D. Bom, R. Andrews, D. Jacques, J. Anthony, B. Chen, M. S. Meier, and J. P. Selegue, "Thermogravimetric Analysis of the Oxidation of Multiwalled

Carbon Nanotubes: Evidence for the Role of Defect Sites in Carbon Nanotube Chemistry," *Nano Letters*, vol. 2, no. 6, pp. 615–619, Jun. 2002.

[110] M. a.M. Motchelaho, H. Xiong, M. Moyo, L. L. Jewell, and N. J. Coville, "Effect of acid treatment on the surface of multiwalled carbon nanotubes prepared from Feâ€"Co supported on CaCO3: Correlation with Fischerâ€"Tropsch catalyst activity," *Journal of Molecular Catalysis A: Chemical*, vol. 335, no. 1-2, pp. 189–198, Feb. 2011.

[111] S. Kundu, Y. Wang, W. Xia, and M. Muhler, "Thermal Stability and Reducibility of Oxygen-Containing Functional Groups on Multiwalled Carbon Nanotube Surfaces: A Quantitative High-Resolution XPS and TPD/TPR Study," *The Journal of Physical Chemistry C*, vol. 112, no. 43, pp. 16 869–16 878, Oct. 2008.

[112] F. A. Abuilaiwi, T. Laoui, M. Al-harthi, and M. A. Atieh, "MODIFICATION AND FUNCTIONALIZATION OF MULTIWALLED CARBON NANOTUBE (MWCNT) VIA," vol. 35, no. 1, pp. 37–48, 2010.

[113] Y. Yun, V. Shanov, Y. Tu, S. Subramaniam, and M. J. Schulz, "Growth mechanism of long aligned multiwall carbon nanotube arrays by water-assisted chemical vapor deposition." *The journal of physical chemistry. B*, vol. 110, pp. 23 920–23 925, 2006.

[114] Y. Li, W. Kim, Y. Zhang, M. Rolandi, D. Wang, and H. Dai, "Growth of single-walled carbon nanotubes from discrete catalytic nanoparticles of various sizes," *Journal of Physical Chemistry B*, vol. 105, pp. 11 424–11 431, 2001.

[115] K. Liu, K. Jiang, C. Feng, Z. Chen, and S. Fan, "A growth mark method for studying growth mechanism of carbon nanotube arrays," *Carbon*, vol. 43, no. 14, pp. 2850–2856, Nov. 2005.

[116] C. Emmenegger, J. Bonard, P. Mauron, and A. Lepora, "S ynthesis of carbon nanotubes over Fe catalyst on aluminium and suggested growth mechanism," vol. 41, pp. 539–547, 2003.

[117] Y. Mo, a.K.M.F Kibria, and K. Nahm, "The growth mechanism of carbon nanotubes from thermal cracking of acetylene over nickel catalyst supported on alumina," *Synthetic Metals*, vol. 122, no. 2, pp. 443–447, Jun. 2001.

[118] L. Pang, J. Saxby, and S. Chatfield, "Thermogravimetric analysis of carbon nanotubes and nanoparticles," *The Journal of Physical Chemicstry*, vol. 97, no. 27, pp. 6941–6942, 1993.

[119] I. D. Rosca, "Oxidation of multiwalled carbon nanotubes by nitric acid," vol. 43, pp. 3124–3131, 2005.

[120] K. a. Wepasnick, B. a. Smith, K. E. Schrote, H. K. Wilson, S. R. Diegelmann, and D. H. Fairbrother, "Surface and structural characterization of multi-walled carbon nanotubes following different oxidative treatments," *Carbon*, vol. 49, no. 1, pp. 24–36, Jan. 2011.

[121] M. G. Pereira and M. A. Z. Arruda, "Article Vermicompost as a Natural Adsorbent Material : Characterization and Potentialities for Cadmium Adsorption," vol. 14, no. 1, pp. 39–47, 2003.

[122] F.-t. Synthesis and B. H. Davis, "Loading of Cobalt on Carbon Nanofibers," pp. 45–73, 2007.

[123] P. F. Rupani, R. P. Singh, M. H. Ibrahim, and N. Esa, "Review of Current Palm Oil Mill Effluent (POME) Treatment Methods : Vermicomposting as a Sustainable Practice," vol. 10, no. 10, pp. 1190–1201, 2010.

[124] J. Coca-Prados and G. Gutiérrez-Cervelló, *Water Purification and Management*, 2011.

[125] X. He, M. Iasmin, L. O. Dean, S. E. Lappi, J. J. Ducoste, and F. L. de los Reyes, "Evidence for fat, oil, and grease (FOG) deposit formation mechanisms in sewer lines." *Environmental science & technology*, vol. 45, no. 10, pp. 4385–91, May 2011.

[126] A. Fakhru'l-Razi, A. Pendashteh, L. C. Abdullah, D. R. A. Biak, S. S. Madaeni, and Z. Z. Abidin, "Review of technologies for oil and gas produced water treatment." *Journal of hazardous materials*, vol. 170, no. 2-3, pp. 530–51, Oct. 2009.

[127] M. C. Cammarota, "A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content," vol. 97, pp. 2195–2210, 2006.

[128] V. Diez, C. Ramos, and J. L. Cabezas, "Treating wastewater with high oil and grease content using an Anaerobic Membrane Bioreactor (AnMBR). Filtration and cleaning assays." *Water science and technology : a journal of the International Association on Water Pollution Research*, vol. 65, no. 10, pp. 1847–1853, Jan. 2012.

[129] M. Haussard, I. Gaballah, N. Kanari, P. de Donato, O. Barrès, and F. Villieras, "Separation of hydrocarbons and lipid from water using treated bark." *Water research*, vol. 37, no. 2, pp. 362–74, Jan. 2003.

[130] D. D. Freire, M. C. Cammarota, and G. L. Santanna, "Biological treatment of oil field wastewater in a sequencing batch reactor." *Environmental technology*, vol. 22, no. 10, pp. 1125–35, Oct. 2001.

[131] K.-E. Jaeger and T. Eggert, "Lipases for biotechnology." *Current opinion in biotechnology*, vol. 13, no. 4, pp. 390–397, Aug. 2002.

[132] L. Cao, *Carrier-bound immobilized enzymes: principles, application and design*, 2006.

[133] G. Bickerstaff, "Immobilization of Enzymes and Cells," vol. 1, Dec. 1996.

[134] Q. Shi, D. Yang, Y. Su, J. Li, and Z. Jiang, "Covalent functionalization of multi-walled carbon nanotubes by lipase," *Journal of Nanoparticle ...*, 2007.

[135] M. V. Naseh, A. A. Khodadadi, Y. Mortazavi, O. A. Sahraei, F. Pourfayaz, and S. M. Sedghi, "Functionalization of Carbon Nanotubes Using Nitric Acid Oxidation and DBD Plasma," pp. 66–69, 2009.

[136] B. Menaa, M. Herrero, V. Rives, M. Lavrenko, and D. K. Eggers, "Favourable influence of hydrophobic surfaces on protein structure in porous organically-modified silica glasses." *Biomaterials*, vol. 29, no. 18, pp. 2710–8, Jun. 2008.

[137] R. Murty, J. Bhat, and P. K. A. Muniswaran, "Hydrolysis of Oils by Using Immobilized Lipase Enzyme : A Review," pp. 57–66, 2002.

[138] G. G. Sándor BESZÉDES, Gábor SZABÓ, "APPLICATION OF THERMAL AND MICROWAVE PRETREAMENTS FOR DAIRY WASTEWATER SLUDGE," *International journal of engineering*, pp. 231–235, 2012.

[139] C. Fang and P. Lai, "Microwave heating and separation of water-in-oil emulsions," *Journal of microwave power and electromagnetic ...*, pp. 46 – 57, 1995.

[140] V. Camila and D. Mayolett, "Treatment of waste-water/oil emulsions using microwave radiation," *Proceedings of SPE International Conference on Health, Safety and Environment in Oil and Gas Exploration and Production*, Mar. 2002.

[141] D. Guzm, P. Flores, T. Rojo, R. MartÄ±, E. C. L, P. D. IngenierÄ±, and I. Mexicano, "Ionic Liquids as Demulsifiers of Water-in-Crude Oil Emulsions : Study of the Microwave Effect," no. 15, pp. 3610–3615, 2010.

[142] V. Camila and D. Mayolett, "Treatment of waste-water/oil emulsions using microwave radiation," *Proceedings of SPE International Conference on Health, Safety and Environment in Oil and Gas Exploration and Production*, Mar. 2002.

[143] M. L. Jayme, D. E. Dunstan, and M. L. Gee, "Zeta potentials of gum arabic stabilised oil in water emulsions," vol. 13, pp. 459–465, 1999.

VITAE

- **Personal Details:**
  - Name: Ammar Jamie
  - Nationality: Sudanese
  - Date of Birth: 13^{th}, February, 1990
  - Email: *ammarjamie@hotmail.com*
  - Permenant Address: Al-Thawra Harra 49, Omdurman, Khartoum, Sudan

- **Educational Background:**
  - 2011 Bachlore of Science, Chemical Engineering, University of Khartoum, Sudan.

- **Publications:**
  - "Immobilization and Enhanced Catalytic Activity of Lipase on Modified MWCNT for Oily Wastewater Treatment". *Ammar Jamie, Ali Al-Shami, Othman Al-Hamouz, Muataz A. Ateih, Zuhair Malaibari.* (To be submitted).
  - "Kinetics Study For the Hydrolysis Of Olive Oil Using Candida Rugosa Lipase". *Ammar Jamie, Ali Al-Shami, Muataz A. Ateih, Zuhair Malaibari.* (To be submitted).
  - "Microwave Pretreatment Effects on Oily Wastewater Hydrolysis Using Immobilized and Free". *Ammar Jamie, Ali Al-Shami, Muataz A. Ateih, Zuhair Malaibari.* (To be submitted).
  - "The Effect of Aspect Ratio of Multi-wall Carbon Nanotubes on Lipase Immobilization". *Ammar Jamie, Muataz A. Ateih, Zuhair Malaibari, Ali Al-Shami.* (To be submitted).